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CIBA FOUNDATION COLLOQUIA ON ENDOCRINOLOGY

VOLUME VI

Hormonal Factors in Carbohydrate Metabolism

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FOREWORD

by

PROFESSOR C. H. BEST, C.B.E., M.D., F.R.C.P., F.R.S.

THE results of well-controlled investigations are not available to provide the answers to many of our more general questions. How much of what is reported in this volume would have been generally available if there had been no Colloquium? How many new experimental approaches to unsolved problems have been or will be formulated as a result of the published and unpublished discussions which this meeting made possible? How much has this Colloquium really helped those of us who were privileged to attend and those who will read this record?

Many of the participants met again during the summer and some of these questions were answered. It was a successful Colloquium and many new studies will arise from it. Misconceptions were removed New knowledge was gained. Further proof that small meetings of keenly interested investigators are valuable, was undoubtedly provided.

A great deal of time and energy and considerable money is required for the organization and conduct of these meetings. All the participants wish to thank The Ciba Foundation and Dr G. E. W. Wolstenholme and his efficient and helpful staff. Our colleague Professor Frank Young was responsible, with Dr. Wolstenholme, for the arrangement of the Scientific Programme and we would like to express to him also our indebtedness and cordial thanks.

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PART I

ENZYME SYSTEMS CONCERNED IN CARBOHYDRATE METABOLISM

ALTERNATIVE ROUTES OF CARBOHYDRATE METABOLISM

Erratum

The plate showing Figs. 1 and 2 (*Conn*) has been placed between pages 12 and 13 in error. Its correct position is between pages 128 and 129.

concentrate of carbo- available to *animal tissues.*

The Glycolytic Route

As regards *anaerobic* breakdown of carbohydrate in animal tissues, there is now little or no opposition to the acceptance of the pathways of the Embden-Meyerhof-Cori glycolytic chain, and a steady accumulation of data for various animal tissues continues to support the importance and widespread occurrence of this type of phosphorylative breakdown (Fig. 1). It is worth making a few points about the more familiar glycolytic system at this stage, in order to contrast this with the alternative routes. First, during the conversion of 1 molecule of hexose to 2 trioses, 2 high energy phosphate bonds are required from 2 moles of ATP.* Similar conversion of 1 hexose unit of glycogen requires 1 mole of inorganic phosphate and

*The following abbreviations are used –
[illegible]

1 mole of ATP. Since no oxidative reaction occurs up to the triose stage, no co-dehydrogenase (DPN or TPN) is required up to that stage; the oxidation of glyceraldehyde-phosphate by its specific dehydrogenase is coupled by DPN with the

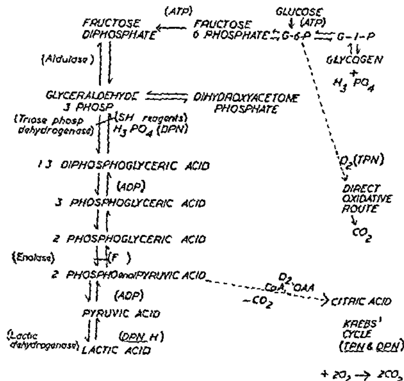


FIG. 1. Glycolytic route, showing its relationship to the HMP-oxidative route.

reduction of pyruvate by lactic dehydrogenase. During these two later stages of the glycolytic cycle, 2 moles of inorganic phosphate are taken up and two high energy phosphate bonds are generated per mole of triose. (1) by the dehydrogenation of diphosphoglyceraldehyde, and (2) by the dehydration of 2-phosphoglyceric acid by enolase. The former reaction is catalysed by a sulphydryl enzyme which is easily blocked by

iodoacetate and other -SH reagents, while enolase is readily poisoned by low concentrations of fluoride ion. In brief, the glycolytic system is a DPN system sensitive to iodoacetate and fluoride, requiring inorganic phosphate and involving ATP or ADP at stages other than the initial hexokinase reaction. These glycolytic enzymes so far discussed are all soluble enzymes, contained in the cytoplasm. It is believed that by a reversal of the glycolytic chain glycogen can be synthesized from lactate, pyruvate or other intermediates, via triose phosphate $\xrightarrow{\text{aldolase}}$ fructose 1:6-diphosphate. This view is in accordance with the incorporation of labelled CO_2 mainly (though not entirely; see Wood, 1951, p. 227) into the 3 and 4 positions of the hexose chain.

It is necessary here to mention some special aspects of fructose metabolism, which have a bearing on alternative pathways of carbohydrate metabolism. Liver and muscle contain in addition to the more familiar type of hexokinase, a specific fructokinase which phosphorylates fructose in the 1-position with the aid of ATP (Cori, Ochoa, Slein and Cori, 1951; Leuthardt and Testa, 1951). Fructose-1-phosphate can also be synthesized from the triose stage by the action of muscle aldolase on a mixture of D-glyceraldehyde and dihydroxyacetone-phosphate (Meyerhof, Lohmann and Schuster, 1936). The reaction has been considered as going to completion, but Dr. de Duve in the following paper will be discussing its reversal as well as other peculiarities of fructose phosphorylation, particularly those in liver tissue, so that it is unnecessary to go into details here, except to point out analogies with the breakdown of pentose phosphate and resynthesis of fructose monophosphate, which will arise at a later stage of the present paper.

Under oxidizing conditions, lactic acid is not produced from pyruvate, and the necessary re-oxidation of the DPN which has been reduced by the triose-phosphate dehydrogenase, is linked by the cytochrome oxidase system with atmospheric oxygen either through the Szent-Gyorgyi system of 4-carbon acids, or else by a specific flavoprotein, DPN-cytochrome c

reductase contained in the mitochondria and microsomes (Hogeboom, 1949), which links reduced DPN with cytochrome c. The further oxidation of pyruvate via the Krebs' cycle to CO_2 and water would require TPN for the *iso*citric dehydrogenase stage and DPN elsewhere.

It has become customary to describe this as the "glycolytic" route of oxidative carbohydrate breakdown. It is probable that this pathway occurs in many tissues, and in muscle probably accounts for the major part of carbohydrate oxidation.

The HMP Oxidative Route: Earlier Work*

In other tissues, and probably to some extent in muscle also, other pathways can lead to carbohydrate oxidation. Although the extent to which they share this function with the glycolytic system is still undefined, the existence of the necessary enzyme chain for the so-called "HMP oxidative route" is now clearly established. Warburg, Christian and Griese (1935) as a result of their studies on the oxidation of glucose-6-phosphate by red blood cells and by Lebedew yeast maceration extract, described the preparation of glucose-6-phosphate dehydrogenase (called by them Zwischenferment). They found that this catalyses the TPN-specific oxidation of its substrate to 6-phosphogluconic acid, which was identified as the final product formed. Inorganic phosphate was not required (Negelein and Haas, 1935). It may be mentioned here that recent work by O. Cori and Lipmann (1952) shows that, as suggested independently on theoretical grounds by Dickens and Glock (1951), the primary product of this dehydrogenation is probably 6-phospho-δ-gluconolactone, the reaction thus being a simple dehydrogenation occurring at C_1 of the gluco-pyranose ring. The reversal of this reaction (reduction of lactone to G-6-P) is made thermodynamically less remote by this representation of the reaction (Cori and Lipmann, 1952), but attempts in our laboratory to demonstrate spectrophotometrically the

* ... I used the phrase

reoxidation of reduced TPN by the enzyme from liver and excess 6-phosphogluconolactone have not so far been successful.

Further breakdown of 6-phosphogluconate was shown to occur in yeast extracts by Warburg, Christian and Griese (1935) and Warburg and Christian (1937); the crude products were partially separated and shown to give the phloroglucinol pentose reaction. Lipmann (1936) had suggested on the basis of the oxygen uptake and CO_2 output, which continued in the presence of bromo-acetate, that oxidation to a 2-ketophosphogluconate might be the primary reaction and that this product might be expected to yield D-arabinose on decarboxylation. Independently, Dickens (1936) observed that separate yeast enzymes effected the oxidation of G-6-P and 6-PG, and described the preparation of a 6-PG-dehydrogenase. This required TPN as coenzyme, and its properties were investigated (Dickens, 1938*a*). The products of the reaction were fractionated as lead and barium salts and analyses showed the presence of ketohexonic acid, pentose and tetronic acid, as their phosphoric esters. On the basis of these results a tentative scheme of carbohydrate degradation was advanced (Dickens, 1938).

D-Arabinose-5-phosphate was not in fact, as Lipmann suggested, attacked further by yeast enzymes, or it was only attacked very slowly, as was also xylose-5-phosphate. Dickens (1938*a*) found instead that D-ribose-5-phosphate was vigorously oxidized in presence of a suitable enzyme preparation from yeast, TPN, and a carrier (phenazine methosulphate) which couples reduced TPN with atmospheric oxygen.

Another preparation, made by Dickens (1938*b*) from dried dialysed Lebedew maceration extract, was found to ferment specifically D-ribose-5-phosphate, and was again almost inert towards the arabinose and xylose phosphates, and also towards the unphosphorylated pentoses. Under anaerobic conditions in the presence of DPN, fermentation of ribose-5-phosphate proceeded to about 90 per cent completion and the products from 1 mole D-ribose-5-phosphate were 1 mole

reductase contained in the mitochondria and microsomes (Hogeboom, 1949), which links reduced DPN with cytochrome c. The further oxidation of pyruvate via the Krebs' cycle to CO_2 and water would require TPN for the *iso*citric dehydrogenase stage and DPN elsewhere.

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* [illegible] I used the phrase [illegible] Cori questioned [illegible] his objection we [illegible]

Recent Progress in the HMP Oxidative Route

Between 1938 and 1949 no further work was done on the mechanism of the HMP oxidative route. Then, making excellent use of the newer techniques of chromatography and identification of sugars by adapted strains of *E. coli*, Cohen and McNair Scott (1949, 1950, 1951) presented a detailed re-investigation, using the same yeast enzyme preparation with phenazine methosulphate as carrier, as used earlier by Dickens. The predicted formation of ribose-5-phosphate from hexose by yeast enzymes was confirmed by both techniques; it was however thought to be accompanied by other pentose phosphates including some D-arabinose-phosphate and there was also chromatographic evidence of the presence of 2-ketogluconate and an acidic reducing substance possibly resembling an *iso*ascorbic acid in its properties. Horecker and his colleagues (Horecker, 1950; Horecker and Smyrniotis, 1950; Horecker, Smyrniotis and Seegmiller, 1951, Horecker and Smyrniotis, 1951*a*, *b*, 1952) have recently made outstanding contributions to this problem With a more highly purified preparation of yeast 6-phosphogluconic acid dehydrogenase, in the presence of TPN, they showed that the first detectable product accumulating from the oxidation of 6-phosphogluconate was the *lævo-rotatory* ketopentose derivative, ribulose-5-phosphate, later an equilibrium mixture was formed, by an enzyme named phosphopentose isomerase, consisting of ribulose-5-phosphate (25 per cent) and ribose-5-phosphate (75 per cent). Both sugars were characterized by derivatives. No evidence of arabinose phosphate formation was reported.

These various results, with some other details of possible reaction-routes, are presented in Fig. 2

It is therefore clear that ribose phosphate is in fact produced by a chain of reactions from glucose, and there is some evidence that the phosphate radical is at position 5, corresponding to the original situation at position 6 in 6-phosphogluconate, isotopic evidence also exists for the elimination of C_1 of the latter as CO_2 (Cohen, 1951)

Lipmann (1936) had already shown that bromoacetate,

CO_2 + 1 mole C_2H_5OH + 1 mole H_3PO_4, together with a *residual product which could not be identified but presumably* resulted from the C_2- residue of the pentose molecule:—



$$C_5H_9O_5.PO_3H_2 + H_2O = C_2H_5OH + CO_2 + H_3PO_4 + C_2H_4O_2$$

The only known route for the production of alcohol by yeast is by the familiar path from triose-phosphate, via pyruvate with decarboxylation of the latter to alcohol and CO_2. This is in accord with the need for DPN, inorganic phosphate, Mg and Mn, and a trace of "activator" (acetaldehyde, glycolaldehyde or hexose diphosphate) observed (Dickens, 1938*b*) Hence the above evidence showed the presence of anaerobic systems which cleave the pentose phosphate molecule into 1 mole triose phosphate leaving 1 mole of a C_2 intermediate (possibly glycolaldehyde, $CH_2OH.CHO$), which did not appear as such but underwent further changes. The probable nature of the product formed from the C_2 residue will be discussed later.

It is noteworthy that only the *phosphorylated* pentose is attacked by cell-free extracts Intact cells do not attack pentose phosphates and are presumed to be impermeable to these phosphorylated substrates, a fact which no doubt assists the retention of these intermediates within the cell.

On the basis of these observations it was suggested (Dickens, 1938*a*) that oxidative degradation of phosphogluconic acid, via ketophosphogluconic acid, gave, instead of the expected D-arabinose-5-phosphate suggested by Lipmann, the corresponding D-ribose-5-phosphate—a reaction involving a Walden *inversion of the glucose molecule at* C_2. *A further suggestion* that continuation of this type of oxidative degradation beyond the ribose stage occurred in yeast requires re-investigation; in extracts of homogenized animal tissues it has been found in our laboratory recently that 5-phospho-D-ribonic acid is not attacked (Glock, 1952*b*), and alternative explanations of the fate of the pentose phosphate are therefore more probable.

liver carcinoma, kidney, bone-marrow, spleen >heart, brain, red cells >Rous sarcoma, skeletal muscle. These estimations are probably only approximate and a more refined assay method has recently been developed (Glock and McLean, 1952*a*, *b*), and is represented diagrammatically in Fig. 3 for the case of liver tissue This result shows that even in the crude

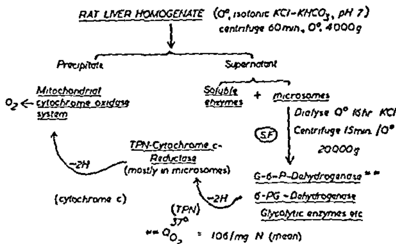


FIG 3 Localization and interactions of dehydrogenases for glucose-6-phosphate and 6-phosphogluconate in the liver cell (Glock and McLean, 1952)

complete soluble-enzyme fraction the enzymic activity is high, and sufficient to compete effectively with the glycolytic system. The probability of such a competition is increased by the fact that the same soluble fraction contains both "glycolytic" and "direct oxidative" enzyme systems. Fig. 3 also shows how in liver, TPN-cytochrome c reductase, contained almost entirely in the microsomes and mitochondria (Hogeboom and Schneider, 1950), can couple the soluble TPN-glucose-6-phosphate-dehydrogenase system with cytochrome c, and thus via the mitochondrial cytochrome oxidase system with atmospheric oxygen. The experimental verification of

which arrests fermentation, has little effect on the HMP oxidative route in yeast extract. Dickens and Glock (1951) showed that even 0·01 Molar iodoacetamide or 0·01 Molar sodium fluoride has very little effect on the activity of glucose-6-phosphate or 6-phosphogluconate dehydrogenases, and that

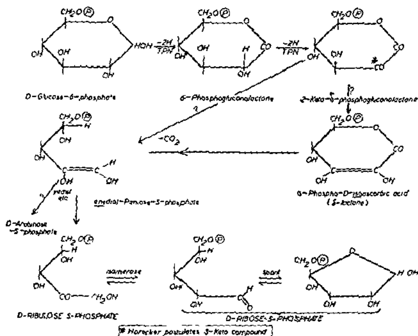
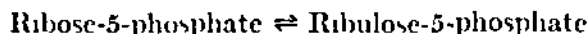


FIG 2. Probable and possible intermediates in the transformation of hexose to pentose.

oxidation of neither substrate required ATP or inorganic phosphate. These facts, together with the specific TPN requirement of the enzymes, serve to differentiate the direct oxidative from the glycolytic route.

Similar enzymes occur in animal tissues (Dickens and Glock, 1950, 1951; Seegmiller and Horecker, 1952), and there is agreement over the distribution of activity of dehydrogenases which in the case of phosphogluconic dehydrogenase may be represented in decreasing order somewhat as follows: yeast, liver,

adenosine, guanosine and ribose-5-phosphate was converted by minced animal tissues, especially liver, into non-pentose phosphoric esters. One of these products was isolated and identified (Waldvogel and Schlenk, 1947) as Robison ester. These authors and Dische (1949) pointed out that the formation of a *mono*phosphorylated hexose was surprising, if its synthesis occurred via the triose-phosphate molecule split off from the pentose phosphate. Recently independent investigations in Dr Cori's laboratory on yeast (Sable, 1952) and in our own on liver (Glock, 1952*b*) have confirmed the anaerobic production of hexose phosphate from ribose-5-phosphate in suitable enzyme extracts Sable has also studied muscle extracts, in which further breakdown of the product does not occur, and this enabled him to identify the first stage as ribulose-5-phosphate, a result which is in agreement with the work of Horecker *et al.* already mentioned on the oxidative breakdown of phosphogluconate. The equilibrium is produced by an enzyme, phosphoriboisomerase, which catalyses the reaction.—


Ribose-5-phosphate ⇌ Ribulose-5-phosphate

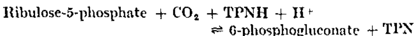
The ketopentose phosphate is then broken down by an enzyme, present in brain and yeast, distinct from muscle aldolase The product consists, in Sable's experiments, of a mixture of hexose diphosphate and the monophosphate which Sable considers to arise from aldolase condensation of two triose phosphate molecules, followed by partial hydrolysis of one of the phosphate groups by phosphatase.

This work is largely in agreement with our own (Dickens and Glock, 1951; Glock, 1952*a*, *b*), but it seems to us improbable that the hexose monophosphate formed in our less crude extracts arises via phosphatase action on fructose-1.6-diphosphate, since (*a*) the latter is not oxidized at all by TPN + liver fractions which readily attack both ribose-5-phosphate and hexose monophosphate, (*b*) these same liver fractions are markedly deficient in fructose-diphosphatase, and (*c*) the

this is clearly shown when the cytochrome c reduction is followed photometrically (Glock and McLean, 1952*a*).

The reduction of glutathione is also coupled in the same (microsome + soluble enzyme) fraction with reduced TPN by means of Meldrum and Tarr's (1935) glutathione-reductase, recently re-investigated by Rall and Lehninger (1952); this enzyme catalyses the reaction TPN H_2 + GSSG ⟶ 2 GSH + TPN. The protection or activation of -SH enzymes by reduced glutathione formed in this way may be a valuable side-property of the direct-oxidative system. This action has also been shown photometrically by my colleagues Drs. G. E. Glock and P. McLean (1952*b*), to whom I am grateful for permission to quote these unpublished observations.

Very recently, the reversal of the enzymatic oxidative decarboxylation of 6-phosphogluconate has been shown by Horecker and Smyrniotis (1952), who have succeeded in demonstrating the fixation of $^{14}CO_2$ into 6-phosphogluconate in the presence of phosphogluconic dehydrogenase.—



$$\text{Ribulose-5-phosphate} + CO_2 + \text{TPNH} + H^+ \rightleftharpoons \text{6-phosphogluconate} + \text{TPN}$$

There is a close formal resemblance between this reaction and the CO_2 fixation occurring with α-ketoglutarate and pyruvate, as studied by Ochoa If this reaction proceeds as written above, it clearly points to ribulose and CO_2 as the *primary* reaction products of phosphogluconate metabolism. As already mentioned, it is not known if the reduction of 6-phosphogluconate to hexose phosphate is enzymatically feasible, although this possibility is suggested by the very similar reduction of the lactone of free gluconic acid which can be made to occur in presence of reduced DNP and glucose dehydrogenase (Strecker and Korkes, 1951).

The Further Metabolism of Pentose Phosphate Formed Oxidatively

Continuing and extending earlier work of Dische (1938), Schlenk and Waldvogel (1946, 1947) reported that the ribose of

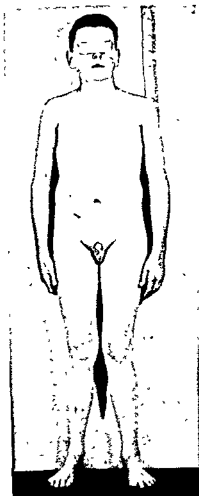


FIG 1 (*Conn*) Photograph of 17-year-old male pituitary dwarf with co-existing diabetes mellitus

[*To face page 12*

yield of hexose monophosphate found with hæmolysed erythrocytes (Dische, 1949) or with purified liver enzymes (Glock, 1952*b*) is clearly about 50 per cent or more higher than could be accounted for by reunion of only the C_3 fragments of pentose (Fig. 4) Fructose appears undoubtedly to be formed,

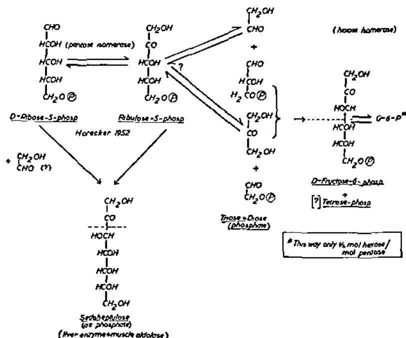


FIG. 4 Ribose-5-phosphate metabolism—some possible routes.

and was detected by both Sable (1952) and Glock (1952*a*, *b*). The latter has estimated the fructose monophosphate by comparison of the extent of TPN reduction by a specific hexosemonophosphate dehydrogenase with the cysteine-carbazole test for fructose, and finds that in liver fractions fructose monophosphate appears to be first formed from ribose-5-phosphate and at a later stage glucose-6-phosphate predominates, due to the slower formation of Robison equilibrium esters by phosphohexose isomerase (Fig. 4). It is generally agreed,

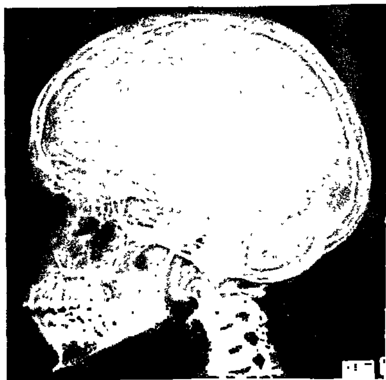


FIG 2 (*Conn*) Photograph of X-ray of skull of subject in Fig 1

however, that free glycolaldehyde does not appear. At present these results cannot be explained satisfactorily. One possible scheme, included in Fig. 4, shows the formation of a 7-C sugar by condensation of glycolaldehyde with pentose phosphate. Glock (1952*b*) has in fact obtained by prolonged incubation of ribose-5-phosphate with the 0·5–0·6 ammonium sulphate saturation precipitable fraction of horse liver, a peak in the photometric cysteine-carbazole test of Dische and Borenfreund (1951) which corresponds closely with that found for a ketoheptose (mannoheptulose, from the avocado pear). After this work was submitted for publication, Horecker and Smyrniotis (1952) published a preliminary account of the purification of rat liver phosphopentose isomerase, which they found, in the presence of crystalline muscle aldolase, synthesized from each 2 moles of ribose-5-phosphate approximately

[illegible] which corres-
[illegible] those of sedo-
[illegible] C_3 moiety was

not determined, but it did not appear as triose phosphate. It is of great interest that both ribulose mono- and di-phosphates (Benson, 1951) and sedoheptulose-monophosphate (Benson, Bassham and Calvin, 1951) accumulate very early in photosynthesis by green plants, the formation of the latter preceding that of hexose monophosphate. This, and the much earlier observation of a ketoheptosephosphate in yeast juice fermentation by Robison, Macfarlane and Tazelaar (1938), suggest that these rare ketoses may possibly play a part in a wide range of biochemical sugar transformations, but the exact mechanism by which hexose monophosphate arises from either of them is still the principal outstanding problem related to the HMP oxidative pathway.

The Occurrence and Significance of Alternative Paths of Carbohydrate Metabolism in Animal Tissues

Shortly after Lundsgaard's discovery of the blocking of glycolysis by iodoacetate, Krebs (1931) observed that the respiration of many tissues was also inhibited unless lactate or

pyruvate was present. Nevertheless Barker, Shorr and Malam (1931) by careful adjustment of the concentration of glycolytic inhibitor were able to separate almost completely the anaerobic glycolysis and carbohydrate oxidation in brain and muscle tissue. Similar results were obtained by thorough washing of brain cortex with Ringer solution. The glucose concentration needed for saturation of respiration (0 02 per cent) was also much lower than for glycolysis (0·2 per cent). The same applies to the phosphate ion concentration (Johnson, 1941) Engelhardt and Barkash (1938) believe that aerobically the activity of phosphohexokinase in phosphorylating fructose monophosphate is inhibited and that oxidation of carbohydrate then proceeds by the direct oxidative route.

Red blood cells have long been known to oxidize glucose-6-phosphate (Warburg and Christian, 1931) and 6-phosphogluconate and ribose-5-phosphate (Dickens, 1938*a*); and ground up brain oxidizes glucose-6-phosphate and 6-phosphogluconate (Dickens, 1936) Recently, Dickens and Glock (1951) and Horecker *et al* (1951) showed the widespread distribution in animal tissues of dehydrogenases for both these substrates, as already mentioned Finally, enzymes breaking down ribose-5-phosphate have recently been described in liver (Dickens and Glock, 1951; Glock, 1952; Horecker and Smyrniotis, 1952; Sable, 1952), brain and skeletal muscle (Sable, 1952) and bone-marrow (Seegmiller and Horecker, 1952). The activity of the oxidative enzymes in liver, at least, is adequate to compete effectively with the glycolytic route (Dickens and Glock, 1951; Glock and McLean, 1952*a*), particularly as they are in the same "soluble enzyme" fraction of the cytoplasm.

The high activity and wide distribution of the enzymes do not of course establish their participation in physiological reactions, but taken with the above indirect evidence they make this probable. As has already been pointed out (Dickens and Glock, 1951) this route would have a dual significance—as a source of pentose for nucleic acids and nucleotides, and as an alternative oxidative path The former function would be expected to be particularly significant for growth, and in

fact some evidence has been brought forward (Orström and Lindberg, 1940) that carbohydrate metabolism in the phase of nuclear development in fertilized sea urchin's eggs, which is oxidative in nature, may proceed by the direct oxidative pathway. The maintenance of -SH groups in the reduced state may also be important in cell-division (cf. Barron, 1949) and could very well be coupled with the reduction of TPN by these

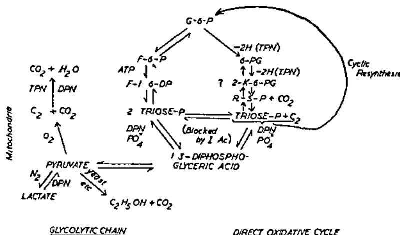


FIG 5 Competition of the HMP-oxidative route with the glycolytic route of carbohydrate metabolism in the cell The triose phosphate molecules are shown separately for convenience, but are presumed to enter a common pool

dehydrogenases in the developing ovum, as in the experiments on liver extracts of Glock and McLean (1952*a*), already described.

It is evident that any such scheme of biological oxidations must at present be purely tentative, but that shown in Fig. 5

interest is the *cyclic* regeneration of hexose monophosphate by a very economical oxidative route, due to the re-condensation of the pentose fragments The details of the last step are

now almost the only obscure point, as has been fully discussed above. It is worth noting here that mechanisms exist for the conversion of ribulose-5-phosphate on the one hand via phosphopentomutase to ribose-1-phosphate (Sable, 1952), which can be incorporated into nucleotides by nucleotide phosphorylase (Kalckar, 1949). Also deoxyribose-phosphate

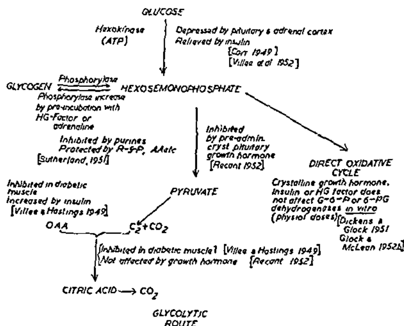


FIG. 6 Tentative scheme of hormonal interrelationships

can be synthesized from the triose phosphate moiety of ribose-5-phosphate via the Racker "DR-aldolase" reaction (Racker, 1952).

Possible Points of Hormone Interaction in Relation to the HMP Oxidative Cycle

Fig. 6 shows some of the key-points at which major hormones controlling carbohydrate metabolism have been described as having their sites of action. It will be noted that

all reactions leading to the formation of hexose monophosphate would be expected to affect both the oxidative and the glycolytic routes. This therefore applies to the insulin control of hexokinase and the effect of hyperglycæmic factor and adrenaline on the phosphorylase reaction. On the other hand, a selective effect on the two paths might be caused by a component of growth hormone, which inhibits the breakdown of hexosemonophosphate to pyruvate (Recant, 1952) though not the metabolism of the C_2 fragment (acetate).

According to Dickens and Glock (1951) some insulin preparations did affect the activity *in vitro* of the dehydrogenases for glucose-6-phosphate and 6-phosphogluconate. The doses used were high, and when the experiments were repeated with physiological amounts in our laboratory, no specific effect whatever of crystalline or amorphous insulin nor of hyperglycæmic factor was observed. Glock and McLean (1952*b*) have also observed that crystalline growth hormone (0·2 mg./ml.) *in vitro* has no effect on these two enzymes. It is very important to see if the HMP-oxidative route of carbohydrate metabolism is also resistant to growth hormone *in vivo*, and experiments with this object in view are now in hand in our laboratory

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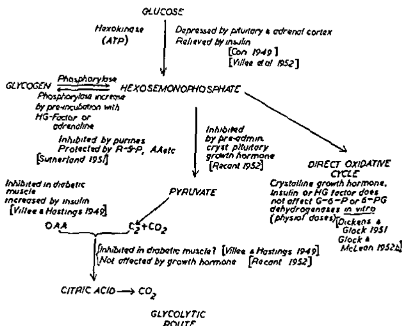


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DISCUSSION

BEST: It is obviously very important that all of us should know this, but it is probably fairly hard going for those of us who are clinicians or physiologists. We would have no difficulty filling the time put aside for this discussion if we limited it to your last slide, but we must not, of course, do that. I would like at this point to encourage discussion on the more biochemical and chemical aspects of the subjects which you have discussed. Dr. Krebs, would you like to introduce this discussion?

KREBS: I would like to ask Dr. Dickens whether he has any views on the physiological significance of these two alternative pathways—are they just alternatives of energy production, or is the second pathway via phosphogluconic acid perhaps a means of producing important cell constituents such as ribose?

DICKENS: I think that direct observations will determine that probably there is some special importance in both, because it obviously has a dual function: (1) the cyclical oxidation of carbohydrate as an end in itself, and (2) the production of pentose phosphates which are [illegible]

[illegible]

which could be coupled with the production of pentose phosphates

required for nucleic acid formation (Rothschild, 1951, *Biochem. Soc. Symposia*, 7, 40). The same sort of necessity may, I suppose, arise in the action of the growth hormone, but I don't profess to know very much about the growth hormone.

G CORI I was wondering whether the term "direct oxidative pathway" is really justified Are you not dealing with a pathway of oxidation as indirect as the one which goes by way of pyruvic acid?

DICKENS I quite agree, Mrs. Cori, on that point. We have recently been investigating what is relatively a much more direct oxidation, which is the formation of gluconic acid from glucose from Harrison's glucose dehydrogenase system This is now known to be activated either by DPN or TPN, and I showed in 1938 that 2-keto-gluconic acid (the unphosphorylated acid) was readily oxidized in kidney slices Since then, Miss D Salmony in our department has been studying oxidation of gluconate and keto-gluconate and finds that these are much more readily oxidized in liver and kidney than most people, I think, realize.

There is an excellent paper by Stetten and Stetten (*J. biol. Chem.*, 1950, 187, 241) using labelled gluconic acid, which shows that part of the carbon at any rate probably finished up in the pentose fraction. I think it would be more reasonable to call that a direct oxidative reaction, and to call the other the hexosemonophosphate oxidative route

LUKENS To what extent could the pentose oxidative pathway be t [illegible] the intact animal by the administration of pentose?

[illegible] and we have been trying to devise [illegible] in the intact animal. A [illegible] *ed* intermediates are attacked One point [illegible] s generally considered as traversing the glycolytic route, [illegible] is certainly a main pathway by which triose can get back to the hexose But the evidence from fixation of carbon is not entirely in favour of that route, there's an excellent review of that by Dr Wood in a recent Ciba Foundation Symposium (*Isotopes in Biochemistry*, p 227), in which he showed that not all of the fixed carbon appears at C_3 and C_4 of the hexose, and it will be interesting to know where the other little bit comes from The most direct observations are on a micro-organism by S S. Cohen (*Nature*, 1951, 168, 746, *Fed Proc*, 1952, 11, 284) who found that by comparing the sugar uptake per cell of *E. Coli* with the activity of the direct HMP-oxidative enzymes, the latter could account for 30 per cent of the oxidation, in agreement with his other experiments on C_1-labelled glucose

C. F. CORI I wonder whether you have thought of an anaerobic pathway for the formation of ribose phosphate, because deoxyribose phosphate is formed by an aldolase type of condensation, which would be an anaerobic process.

DICKENS That's a very interesting point, I think it is quite likely that the system could function anaerobically by re-oxidation of the co-enzyme taking place at the expense of some other mechanism

C. F. CORI Another question I would like to ask is whether the C_2 fragment which is formed in the degradation of ribose phosphate has been identified in your laboratory?

DICKENS: The only thing that I can say definitely about that is that we and many workers are agreed that glycolaldehyde, which is the obvious C_2 intermediate, is not formed. If it is formed, it immediately

α-glucose and β-glucose in the liver?

DICKENS I am afraid we have no information at all on this point.

YOUNG· Can Dr. Dickens throw any light on the biogenesis of ascorbic acid on the basis of these reactions?

DICKENS. I'm going to counter that by asking if the so-called diabetogenic action of dehydro*iso*ascorbic acid is possibly related to this mechanism? It seems a little unlikely that *l*-ascorbic acid is formed by this type of route, because it is not the sort of molecule which one would expect to arise from glucose in this way On the other hand,

you? Have you in fact investigated the possibility that the glucose may be oxidized at carbon 6 and reduced at carbon 1?

oxidation at the other end (C6) of the glucose molecule. It is certainly quite a possibility

NATURE AND INTRACELLULAR DISTRIBUTION OF THE ENZYMES CONCERNED IN THE METABOLISM OF THE HEXOSES IN THE LIVER

CHRISTIAN DE DUVE

Two facts are slowly emerging from the numerous studies which have been concerned in recent years with the hormonal control of carbohydrate metabolism in the liver.—

(1) The enzymatic reactions linking the hexoses, hexosephosphates and glycogen form a central crossroad, on which most hormones are either known or suspected to exert a direct action.

(2) The spatial organization of the enzyme systems involved may play an important part in many of these regulatory mechanisms.

These two facts have prompted us to investigate more closely the nature and intracellular distribution of these systems, as a preliminary to an eventual study of their susceptibility to hormonal influences. The present report will be devoted mainly to a summarized account of the results which have been obtained to date by different workers in this laboratory, including, F. Appelmans, H. Beaufays, J. Berthet, L. Berthet, H G. Hers, T Kusaka, C. A Vuylsteke, R Wattiaux and myself Many of these results are still unpublished and I am indebted to my co-workers for letting me use their data.

Metabolic Pathways and Enzyme Systems

Fig 1 summarizes the main reactions involved in the interconversion of hexoses and glycogen in liver tissue.

The Hexokinases

The only liver hexokinase to be fairly well characterized is *fructokinase*, which has been studied by several authors (Slein,

Cori and Cori, 1950; Vestling, Mylroie, Irish and Grant, 1950; Leuthardt and Testa, 1950*a*, *b*, 1951; Cori, Ochoa, Slein and Cori, 1951; Staub and Vestling, 1951; Hers, 1952*a*, *b*) The enzyme from beef liver has been partly purified by Hers (1952*a*). It is inactive on aldohexoses and on fructose-phosphates, but, as was first shown by Leuthardt and Testa (1950*a*, *b*), phosphorylates other ketohexoses such as sorbose and tagatose. The name *ketohexokinase*, proposed by Leuthardt and Testa (1950*a*, *b*) would therefore seem to be more appropriate. The enzyme requires magnesium and is strongly activated by potassium ions, which act by increasing the affinity of the enzyme for a Mg-ATP complex (Hers, 1952*b*). The product of the reaction is fructose-1-phosphate (Slein *et al.*, 1950, Cori *et al.*, 1951; Leuthardt and Testa, 1951; Staub and Vestling, 1951; Hers, 1952*a*), an ester which accumulates in the liver of fructose-fed animals (Kjerulf-Jensen, 1942)

Very little is known concerning the *glucokinase* of liver. Such an enzyme is undoubtedly present in hepatic tissue, but its activity in homogenates or extracts is often surprisingly low and is easily lost in the course of fractionation (Slein *et al.*, 1950, Vestling *et al*, 1950, Hers, Berthet, Berthet and Duve, 1951; Long, 1952, Hers, 1952*a*) Preliminary experiments have however shown that a considerable activation of the enzyme can take place in some cases, but it has not yet been possible to define the conditions under which it can be made to occur in a reproducible manner. It is not yet known with certainty whether liver glucokinase phosphorylates glucose in the 6 position like other glucokinases, nor whether it or a separate enzyme is responsible for the *mannokinase* activity of the tissue

The existence of a *galactokinase* in liver has been demonstrated by Bacila (1948) and confirmed in this laboratory. Here again, curious phenomena occur and it has repeatedly been found that homogenates with a very low galactokinase activity may yield active fractions From the observation of Kosterlitz (1943) that galactose-1-phosphate accumulates in

PARTICULATE GLYCOGEN
?
1-6 GLYCOGEN
(18) (17)
1-4 GLYCOGEN
+
P
(15)
(14a) (14b)
(16)
(7)
ATP
GALACTOSE (1) GALACTOSE-1-P (9) GLUCOSE-1-P
GLUCOSE
(7)
GLUCOSE (2) GLUCOSE-6-P
(19)
P
MANNOSE (3) MANNOSE-6-P (10)
(8)
MICROSOMES
FRUCTOSE?
FRUCTOSE (4) FRUCTOSE-1-P (11) FRUCTOSE-6-P
ADP
(20)
ATP (5) ADP
P
FRUCTOSE-1-6-DI-P
(12)
GLYCERALDEHYDE + DIOXYACETONE-P + GLYCERALDEHYDE-3-P
(13)
ATP
(6)
ADP
GLUCOGENIC MATERIAL
ADP
ADP + P
LACTATE (22)
ATP
ATP
OXIDIZABLE MATERIAL
ADP + P
(23) $CO_2 + H_2O$
ATP
(21)
P
MITOCHONDRIA

FIG. 1.

the liver of galactose-fed animals, and in analogy to the reaction studied in yeast by Trucco, Caputto, Leloir and Mittelman (1948), and by Wilkinson (1949), it may be assumed that the product of the galactokinase reaction is galactose-1-phosphate.

6-Phosphofructokinase is present in liver, but has not been studied so far. It is not known whether hepatic tissue contains a 1-phosphofructokinase.

Interconversion of Hexose-Phosphates

Phosphoglucomutase and Lohmann's *phosphohexoisomerase* have been known for a long time to be present in liver but have not been specially studied in this tissue. Neither the *galactowaldenase* discovered in yeast by Caputto, Leloir, Cardini and Paladini (1950) nor the *phosphomannose-isomerase* identified by Slein (1950) in rabbit's muscle have yet been evidenced in liver, but it is fairly probable that both enzymes are present

---

FIG 1. Pathways of interconversion of hexoses and glycogen

A *Phosphokinases*

(1) Galactokinase
(2) Glucokinase
(3) Mannokinase ?
(4) Ketohexokinase (fructokinase).
(5) 6-Phosphofructokinase.
(6) Glyceraldehyde-kinase

B *Enzymes acting on interconversion of hexose-phosphates*

(7) Phosphoglucomutase
(8) Phosphohexoisomerase
(9) Galactowaldenase ?
(10) Phosphomannose-isomerase ?
(11) Phosphofructomutase ?
(12) Aldolase
(13) Phosphotriose-isomerase

C *Enzymes acting on glycogen*

(14) Phosphorylase
 (a) Active form.
 (b) Inactive form
(15) Phosphorylase-inactivating enzyme
(16) Phosphorylase-reactivating system
(17) Amylo-1-6-glucosidase (unbranching factor)
(18) Branching factor

D *Phosphatases*

(19) Glucose-6-phosphatase
(20) Hexose-diphosphatase
(21) Acid phosphatase

E *Associated systems*

(22) Glycolytic system
(23) Oxidative phosphorylations

and are involved in the formation of hepatic glycogen from galactose and mannose respectively. Uridine-diphosphate-glucose, the coenzyme of galactowaldenase, has been found in various animal tissues, including liver, by Caputto *et al.* (1950), and Caputto and Trucco (1952) have recently reported the presence of the enzyme itself in the mammary gland of lactating rats. On the other hand, Cook (1952) has shown that mannose is converted into liver glycogen without significant splitting of the molecule into smaller fragments.

The further conversion of fructose-1-phosphate is of particular interest. It has been shown by Cori *et al.* (1951) that liver homogenates convert fructose-1-phosphate into glucose by way of hexose-6-phosphate They found this reaction to be magnesium-dependent and to be inhibited by fluoride and postulated the existence of a *phosphofructomutase*, catalysing the formation of fructose-6-phosphate from fructose-1-phosphate. They were unable to rule out the alternative possibility that fructose-1-phosphate should be first converted to glucose-1-phosphate through a 1-phosphohexoisomerase.

Hers and Kusaka have investigated the metabolism of synthetic and enzymatic fructose-1-phosphate by liver tissue. They confirmed the basic findings of Cori *et al.* (1951), but were unable to obtain any evidence indicating the existence of a phosphofructomutase (or of a 1-phosphohexoisomerase). According to their results, fructose-1-phosphate is first split by *aldolase* into glyceraldehyde and phosphodioxyacetone; the latter is partly isomerized into phosphoglyceraldehyde by *phosphotriose-isomerase* and the two phosphotrioses condense, again through aldolase, into fructose-1-6-diphosphate, which is finally dephosphorylated into fructose-6-phosphate by *hexose-diphosphatase*.

The possibility of such a pathway was first demonstrated by Meyerhof, Lohmann and Schuster (1936) who showed that yeast aldolase catalyses the formation of fructose-1-phosphate from fructose-diphosphate and d-glyceraldehyde. According to these authors, the reaction is irreversible. In the present system, it is able to progress in the reverse direction, thanks

to the hexose-diphosphatase which removes fructose-diphosphate as it is formed, thus displacing the equilibrium of the whole system in favour of fructose-1-phosphate disappearance. The strict magnesium-dependency of this enzyme and the fact that it is inhibited by fluoride (Gomori, 1943) explain the findings of Cori *et al.* (1951).

The facts arguing in favour of this mechanism being the main pathway of fructose-1-phosphate breakdown in liver tissue and against the existence of a phosphofructomutase include the following (1) Hepatic homogenates do not convert fructose-6-phosphate into fructose-1-phosphate, although the thermodynamic data indicate that the equilibrium is in favour of the latter; (2) Activation of the system with magnesium is always accompanied by the production of inorganic phosphate in quantities at least equal to the amount of hexose-6-phosphate formed; (3) Whole, freshly prepared homogenates of liver tissue form considerable quantities of glyceraldehyde, when incubated either aerobically or anaerobically with fructose-1-phosphate Unless special precautions are taken, this process easily escapes detection, since glyceraldehyde is rapidly destroyed by the barium hydroxide used to deproteinize the mixture and behaves like glucose in the copper reduction test (Nelson, 1944, method). If deproteinization is effected by adding the zinc sulphate first and barium hydroxide afterwards, and if the reaction with the copper reagent is carried out during 2 minutes at 80°, the free trioses are estimated specifically *

Fig. 2 illustrates in diagrammatic form the results of a complete balance performed by Hers and Kusaka. Ten micromoles of synthetic fructose-1-phosphate were incubated during one hour at 30° and at *p*H 8 in the presence of magnesium ions with an enzyme system isolated from rat liver according to the method described by Cori *et al* (1951) At the end of the incubation period, 3·6 micromoles of fructose-1-phosphate had been converted into a mixture of triose, fructose, inorganic

*Fructose reacts to some extent under those conditions and a small correction has to be made when this sugar is present in the mixture

and are involved in the formation of hepatic glycogen from *galactose and mannose respectively. Uridine-diphosphate-*glucose, the coenzyme of galactowaldenase, has been found in various animal tissues, including liver, by Caputto *et al.* (1950), *and Caputto and Trucco (1952) have recently reported the* presence of the enzyme itself in the mammary gland of lactating rats. On the other hand, Cook (1952) has shown that mannose is converted into liver glycogen without significant splitting of the molecule into smaller fragments.

The further conversion of fructose-1-phosphate is of particular interest. It has been shown by Cori *et al.* (1951) that liver homogenates convert fructose-1-phosphate into glucose by way of hexose-6-phosphate. They found this reaction to be magnesium-dependent and to be inhibited by fluoride and postulated the existence of a *phosphofructomutase*, catalysing the formation of fructose-6-phosphate from fructose-1-phosphate They were unable to rule out the alternative possibility that fructose-1-phosphate should be first converted to glucose-1-phosphate through a 1-phosphohexoisomerase.

Hers and Kusaka have investigated the metabolism of synthetic and enzymatic fructose-1-phosphate by liver tissue. They confirmed the basic findings of Cori *et al.* (1951), but were unable to obtain any evidence indicating the existence of a phosphofructomutase (or of a 1-phosphohexoisomerase). According to their results, fructose-1-phosphate is first split by *aldolase* into glyceraldehyde and phosphodioxyacetone; the latter is partly isomerized into phosphoglyceraldehyde by *phosphotriose-isomerase* and the two phosphotrioses condense, again through aldolase, into fructose-1-6-diphosphate, which is finally dephosphorylated into fructose-6-phosphate by *hexose-diphosphatase.*

The possibility of such a pathway was first demonstrated by Meyerhof, Lohmann and Schuster (1936) who showed that yeast aldolase catalyses the formation of fructose-1-phosphate from fructose-diphosphate and d-glyceraldehyde. According to these authors, the reaction is irreversible. In the present system, it is able to progress in the reverse direction, thanks

to the hexose-diphosphatase which removes fructose-diphosphate as it is formed, thus displacing the equilibrium of the whole system in favour of fructose-1-phosphate disappearance. The strict magnesium-dependency of this enzyme and the fact that it is inhibited by fluoride (Gomori, 1943) explain the findings of Cori *et al.* (1951).

The facts arguing in favour of this mechanism being the main pathway of fructose-1-phosphate breakdown in liver tissue and against the existence of a phosphofructomutase include the following: (1) Hepatic homogenates do not convert fructose-6-phosphate into fructose-1-phosphate, although the thermodynamic data indicate that the equilibrium is in favour of the latter; (2) Activation of the system with magnesium is always accompanied by the production of inorganic phosphate in quantities at least equal to the amount of hexose-6-phosphate formed; (3) Whole, freshly prepared homogenates of liver tissue form considerable quantities of glyceraldehyde, when incubated either aerobically or anaerobically with fructose-1-phosphate. Unless special precautions are taken, this process easily escapes detection, since glyceraldehyde is rapidly destroyed by the barium hydroxide used to deproteinize the mixture and behaves like glucose in the copper reduction test (Nelson, 1944, method). If deproteinization is effected by adding the zinc sulphate first and barium hydroxide afterwards, and if the reaction with the copper reagent is carried out during 2 minutes at 80°, the free trioses are estimated specifically.*

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phosphate and hexose-6-phosphates, containing traces of triose-phosphates and of fructose-diphosphate. The diagram shows to scale the fate of the carbon and phosphate moities of the disappeared fructose-1-phosphate, together with the assumed pathways. It is clear that the main pathway is the

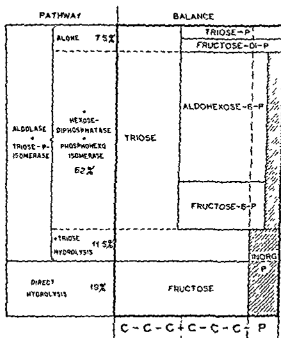


FIG. 2. Fate of fructose-1-phosphate incubated with rat liver fraction.

one described, with two side-reactions due to the splitting of fructose-1-phosphate and triose-phosphates, presumably by hexose-diphosphatase. The enzyme preparation showed a very low hexose 6-phosphatase activity when incubated with an excess of glucose-6-phosphate, which accounts for the virtual absence of free glucose.

Obviously, the evidence presented is only presumptive, and conclusive proof that the liver does not contain a phospho-

fructomutase can only be obtained by following the fate of isotopic fructose *in vivo*. According to the scheme presented, one should expect a high degree of randomization between the 1 and 6 positions in the glucose formed from fructose labelled in the 1 position.

Additional information is needed before the further fate of the glyceraldehyde formed from fructose-1-phosphate can be ascertained. In all probability, it is phosphorylated and enters the system in this manner A fraction has been isolated from high-speed supernatants of rat liver homogenates, which converts d-glyceraldehyde into fructose-1-phosphate in the presence of ATP. Two molecules of glyceraldehyde disappear for each molecule of fructose-1-phosphate formed. Presumably glyceraldehyde is phosphorylated by ATP to 3-phosphoglyceraldehyde. The latter is isomerized in phosphodioxyacetone, which condenses with a second molecule of glyceraldehyde to form fructose-1-phosphate. These results show that the liver possesses *glyceraldehyde-kinase* activity. It remains to be seen whether a specific enzyme is involved.

Another possible pathway of fructose-1-phosphate disappearance would be by way of a direct phosphorylation to fructose-diphosphate by a 1-phosphofructokinase, an enzyme which has been reported by Cori *et al.* (1951) to be present in muscle, but not in brain tissue. Except that aldolase would be short-circuited in this mechanism, the last steps would be the same as in the scheme described and an additional molecule of ATP would also be consumed per molecule of fructose converted to hexose-6-phosphate The results obtained so far are not in favour of this second scheme.

Formation and Breakdown of Glycogen

Our knowledge concerning the reactions involved in the synthesis and breakdown of liver glycogen is due almost exclusively to the work of the Coris and their co-workers They demonstrated the essential rôle of *phosphorylase* and showed that this enzyme catalyses the reversible phosphorolytic splitting of terminal α-1-4 glucosidic linkages (Cori and Cori, 1938,

Cori, Cori and Schmidt, 1939; Cori and Cori, 1943; Hestrin, 1949). Two additional enzymes are needed for the formation and splitting of the 1-6-linkages responsible for the branching of the glycogen molecule. The unbranching enzyme has been recently studied in muscle by Cori and Larner (1951) who showed that it possesses the properties of an *amylo*-1-6-*glucosidase*, acting by hydrolytically detaching one glucose unit from the side chains which have been completely denuded by phosphorylase. Little is known concerning the *branching factor*, which according to recent results of Larner (1952) appears to be an isomerizing enzyme with the properties of a transglucosidase.

In the course of studies on the mechanism of action of adrenaline and glucagon (H-G factor), Sutherland and Cori (1951) have found that the level of active phosphorylase in liver tissue is conditioned by two opposing systems. One of these is an enzyme which converts phosphorylase into an inactive form Sutherland (1951*a*) has been able to purify liver phosphorylase and to separate it from the inactivating enzyme. Using purified systems he has shown that the latter is inhibited by 0·1 M fluoride and by small amounts of adenosine-5-phosphate and of some related compounds, and that it is activated by various purines, including caffeine (Sutherland, 1951*b*, *c*). The nature of the reactivating system is still obscure. Its activity is suppressed when the cellular structure is disrupted by freezing or homogenization. The glycogenolytic agents shift the balance between the two systems in favour of the reactivation of phosphorylase. Since they are entirely inactive on frozen slices or on homogenates and do not inhibit the inactivating enzyme, Sutherland supposes that they act by stimulating the reactivating system.

This system has also been studied in this laboratory with results which fully confirm those of Sutherland's. Dr. Vuylsteke and I have found that if 0·1 M fluoride is added to liver slices which have been depleted of phosphorylase by a preliminary incubation, a rapid reactivation of the enzyme takes place. The same phenomenon has been observed by Suther-

land (1951*b*, *c*). Presumably fluoride acts simply by inhibiting the inactivating enzyme and furnishes a means whereby the reactivating system can be studied. Amongst other data, we have found that the rate of reactivation is greater in air than in nitrogen and still greater in oxygen and that it is enhanced by dinitrophenol. Contrary to that of glucagon, the effect of dinitrophenol adds to that of fluoride. Fig. 3 illustrates

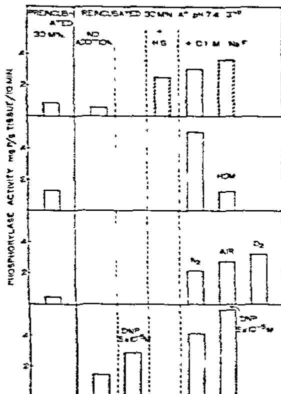


FIG. 3. Reactivation of liver phosphorylase. Four different experiments are described. The ordinates refer to the phosphorylase activity, measured at pH 6 with glucose-1-phosphate, glycogen and fluoride, of the homogenates prepared from slices incubated as described.

HG = 12 μg of purified glucagon.
HOM = homogenized before reincubation.
DNP = dinitrophenol.

these results. From the rapidity with which these reactions occur, it would seem that the turnover of phosphorylase in liver tissue must be very high.

Dephosphorylation of Hexose-Phosphates

Glucose-6-phosphatase has been studied by Swanson (1950)

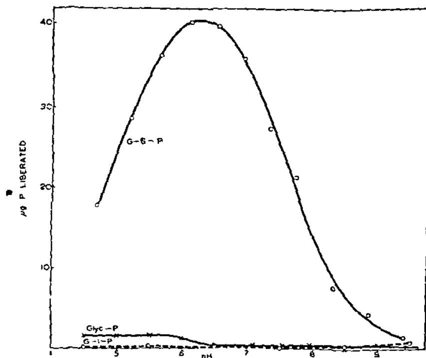


FIG. 4. Specificity of glucose-6-phosphatase. Effect of *p*H on phosphatase activity of washed rat-liver microsomes on 0.008 M glucose-6-phosphate (G-6-P), glucose-1-phosphate (G-1-P) and β-glycerophosphate (Glyc-P)

and in this laboratory (Duve, Berthet, Hers and Dupret, 1949; Hers and Duve, 1950; Hers *et al.*, 1951). The enzyme is associated with an insoluble structure and has not yet been obtained in soluble form. It can be partly purified by agglutination of the particles at *p*H 5 in the cold or better by differential centrifugation of the microsome fraction. By this

method it is possible to obtain preparations which are practically free of phosphoglucomutase, phosphohexoisomerase and acid phosphatase. As shown in Fig. 4, such preparations are inactive towards glucose-1-phosphate and β-glycerophosphate and highly active towards glucose-6-phosphate.

More recent investigations by Beaufays have shown that

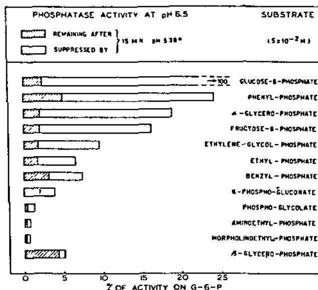


FIG 5 Specificity of glucose-6-phosphatase Action of washed rat-liver microsomes on various phosphate esters at *p*H 6 5

the specificity is not as narrow as was first believed Fig. 5 summarizes the results of an experiment performed on washed microsomes from rat liver The enzyme was incubated during 10 minutes at *p*H 6·5 and 37° in the presence of equivalent amounts of the various substrates mentioned. In order to evaluate the contribution of acid phosphatase to the observed activities, similar tests were performed with the same preparation preincubated during 15 minutes at *p*H 5 and 37° and

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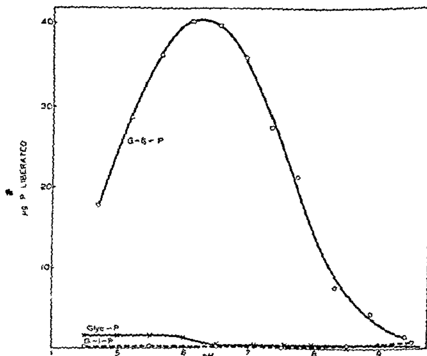


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Coupled Reactions Involving Phosphate and the Adenylic System

The reactions represented in Fig. 1 are intimately associated with the general phosphate metabolism of the liver cell. ATP is used up by the hexokinases and "glyceraldehyde-kinase." Inorganic P is liberated by glucose-6-phosphatase, hexose-diphosphatase and during the synthesis of glycogen by phosphorylase. It is taken up by the phosphorolysis of glycogen. The associated reactions whereby ADP and inorganic P recombine either anaerobically or aerobically have also been schematically depicted in Fig 1.

Intracellular Distribution of the Enzyme

The main instrument for the study of the intracellular distribution of enzymes is the method of differential centrifugation worked out by Claude (1946*a*, *b*) and perfected by Hogeboom, Schneider and Pallade (1948). In this method, the tissue is first homogenized in cold isotonic or hypertonic sucrose and separated by differential centrifugation into various particulate fractions and a final supernatant, which are then analysed for their enzymatic content. Provided various causes of error are correctly eliminated, it is possible in this manner to obtain information concerning the distribution of enzymes between the cellular structures which resist the homogenizing procedure and are separable by centrifugation Data relative to the finer organization of the cell cannot be derived from such experiments.

Using the method of centrifugation, we have studied the intracellular distribution of a number of the enzymes mentioned in this report (Hers *et al*, 1951) Except for glucose-6-phosphatase, which is entirely bound to submicroscopic particles (microsomes), practically all the enzymes involved in the interconversion of the hexoses, glycogen and lactic acid appear to be present in the cell in a readily soluble form This has been demonstrated in a quantitative manner for phosphoglucomutase, hexose-diphosphatase and, by Kennedy and Lehninger (1949), for aldolase. Studies on

brought back to pH 6·5. This treatment destroys glucose-6-phosphatase completely and does not affect acid phosphatase. We are indebted to Dr. Desjobert from Paris for his generous gift of synthetic substrates.

The most surprising of these results is the relatively high activity of the enzyme on fructose-6-phosphate. That a direct action is involved has been verified by measurements of the fructose set free, by estimations of the phosphohexoisomerase activity of the preparation, which was very low, by selective inactivation of glucose-6-phosphatase (see Fig. 5) and by substrate competition experiments.

These results are difficult to reconcile with the fact that very little free fructose is formed from fructose-6-phosphate by liver slices or hepatic homogenates (Cori *et al.*, 1951; Duve *et al.*, 1949) No ready explanation can be given for this discrepancy, but it must be pointed out that glucose-6-phosphatase shows peculiar properties, some of which seem to be related to its particulate nature. The enzyme is very unstable in complete homogenates, where it is protected by phenols. It may be very stable in thoroughly washed microsomes, in which case addition of the soluble fraction increases its rate of inactivation. Agglutination of the particles at pH 5 or freezing and thawing labilize the enzyme and may also alter its specificity. Preparations have been obtained which, after having been frozen and thawed several times, were almost equally active on α-glycerophosphate and on glucose-6-phosphate.

Despite these difficulties, there seems to be little doubt that glucose-6-phosphatase is the enzyme actually involved in the formation of free glucose by liver tissue. It exerts a definitely preferential action on glucose-6-phosphate and is found only in liver and kidney (Hers and Duve, 1950), i.e., in the two organs which are known to manufacture glucose in the body.

Hexose-diphosphatase has been studied by Gomori (1943) and by Roche and Bouchilloux (1950). The enzyme appears to be specific for fructose-1-6-diphosphate and shows an absolute requirement for magnesium ions.

a soluble form which is the real substrate of the glycogenolytic enzymes. The latter possibility appears to be more probable, since it has been found that adrenaline affects mainly the TCA-soluble fraction in muscle glycogen (Bloom, Schumpert and Lewis, 1950).

These various results have been schematically summarized in Fig. 1. In addition to the recognized particulate entities, which are clearly indicated in this figure, two areas have been enclosed in broken lines suggesting the possible existence of labile structures. One contains the hexokinase system and symbolizes our lack of knowledge concerning these enzymes rather than any positive information. The other contains the glycogen-phosphorylase system.

In this case, the experimental data are a little more definite. In the first place, there is the fact, already mentioned above, that freezing or homogenizing liver tissue completely abolishes its ability to respond to the glycogenolytic agents and to reactivate phosphorylase. The finer organization of the cell appears to be necessary for this process, but this is by no means certain, since dilution or destruction of an essential component might also be involved.

Another argument, resting on even more tenuous ground, is derived from some observations made by Dr Vuylsteke and myself which suggest that when phosphorylase is reactivated in liver slices under the influence of glucagon, it becomes at the same time inaccessible to glucose-1-phosphate. Fig. 6 illustrates an experiment of this type. Liver slices were incubated during 10 minutes in the presence of glucose-1-phosphate and fluoride at *p*H 6 (optimal conditions for glycogen synthesis) before and after a period of preincubation with or without glucagon (H-G). A similar set of slices were treated in exactly the same manner except that they were quickly frozen and thawed immediately before the phosphorylase test was conducted. The inorganic phosphate liberated was measured. It is seen that the frozen slices show the drop in phosphorylase which occurs during the preincubation period as well as the presence of extra-phosphorylase in the slices

phosphorylase were complicated by the rapid inactivation o[f] this enzyme, but since as much as 80 per cent could be re[-] covered in the final supernatant after a rapid separation of al[l] the particulate entities, this conclusion would seem to hol[d] true for phosphorylase as well.

High-speed supernatants of liver homogenates were foun[d] to contain a large proportion of the glucokinase and fructo[-] kinase activities. In the presence of fluoride, the reactio[n] proceeded readily to the stage of phosphoglyceric acid, indi[-] cating the presence of phosphofructokinase and other glyco[-] lytic enzymes. As shown by Le Page and Schneider (1948) the whole glycolytic activity of liver homogenates is recovere[d] in the final supernatant.

Owing to the difficulty of estimating hexokinases quanti[-] tatively in complex systems, no conclusion can be drawn as to the exact proportion of the enzymes present in soluble form. Recent results obtained in the study of glucokinase and of galactokinase indicate that the activity of these enzymes is subject to factors which are not fully controlled under the experimental conditions and that structural entities may be involved. It has also been found in recent investigations that the "glyceraldehyde-kinase" activity is essentially concen- trated in the soluble fraction.

The fact that glucose-6-phosphatase is quantitatively bound to microsomes is suggestive. In crude homogenates, this en- zyme is a very disturbing factor and renders a net uptake of glucose almost impossible, unless fluoride is added to inhibit the phosphatase Since intact liver cells readily take up glucose, it is reasonable to suppose that their glucose-6-phos- phatase activity is held in check under certain conditions and the fact that the enzyme is firmly bound to a structure may be relevant to this process.

Finally, it should be recalled here that a large fraction of the liver glycogen can be separated in particulate form by high-speed centrifugation (Lazarow, 1942; Claude, 1946*b*). It is not known whether particulate glycogen is directly suscep- tible to enzymatic action or whether it is in equilibrium with

a soluble form which is the real substrate of the glycogenolytic enzymes. The latter possibility appears to be more probable, since it has been found that adrenaline affects mainly the TCA-soluble fraction in muscle glycogen (Bloom, Schumpert and Lewis, 1950).

These various results have been schematically summarized in Fig. 1. In addition to the recognized particulate entities, which are clearly indicated in this figure, two areas have been enclosed in broken lines suggesting the possible existence of labile structures. One contains the hexokinase system and symbolizes our lack of knowledge concerning these enzymes rather than any positive information. The other contains the glycogen-phosphorylase system.

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incubated with H-G. The decrease is also apparent in the intact slices, but not the relative increase in the H-G-treated slices.

We have been tempted to attach some significance to these results because the existence of a barrier between the reactivated phosphorylase and glucose-1-phosphate would explain the fact that the glycogenolytic agents always favour glyco-

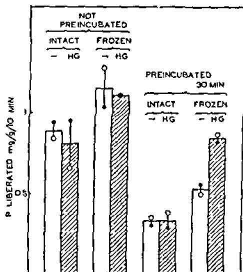


FIG. 6. Inaccessibility of HG-reactivated phosphorylase to glucose-1-phosphate? Means of duplicate values (represented individually by small spheres).

genolysis, even in a liver which is actively synthesizing glycogen from glucose, and because we have been unable, on the other hand, to find any evidence that these agents modify the equilibrium conditions of the phosphorylase reaction. However, we are aware that many other explanations can be given for the observed results, and in our opinion they have only the value of a slight indication.

In Fig. 1, allusion has also been made to the rôle of the mitochondria in the metabolism of hexoses and glycogen. Although

they appear to contain none of the enzymes directly concerned with this metabolism, they may nevertheless play an important part in the regulation of their activities by controlling the levels of ATP and inorganic phosphate within the liver cell. It is now a well established fact that the mitochondria are the main site of oxidative phosphorylations. As such they are presumably involved in supplying ATP to the hexokinase system and in withdrawing inorganic phosphate from the *phosphorylase system, thereby favouring glycogen synthesis.* An inhibition of such a mechanism might well account for the well known phenomenon of anoxic hyperglycæmia.

In previous experiments from this laboratory, special attention has been paid to the mitochondria-linked acid phosphatase of liver (Duve, Berthet, Berthet and Appelmans, 1951; Berthet and Duve, 1951; Berthet, Berthet, Appelmans and Duve, 1951) The peculiar character of the bound enzyme made it possible to study some of the structural and osmotic properties of its particulate support. Although not directly relevant to the problems at hand, these experiments are re*called here, because conclusive proof has now been obtained* that acid phosphatase belongs to a population of granules which are different both from the cytochrome-oxidase-bearing mitochondria and from the glucose-6-phosphatase-containing microsomes. Liver tissue therefore contains at least one additional particulate entity besides those already recognized. Its function still remains to be explored

Conclusions

Summarizing the data which have been gathered so far, we find that the components of the systems involved in the interconversion of hexoses and glycogen are distributed between at least four distinct cellular phases:—

(1) Particulate glycogen, the significance of which is unknown

(2) The glucose-6-phosphatase-bearing microsomes which control the glucose output of the liver cell.

(3) The mitochondria which govern *to* a large extent the turnover of inorganic phosphate and of ATP.

(4) The soluble fraction which contains practically all the enzymes concerned.

This partition is fairly well preserved in homogenates and represents therefore the minimum level of organization of these systems within the living cell. As such it may serve as a first basis for any attempt to reconstruct the self-regulatory mechanisms which are at work in the intact organ. It would be premature to speculate on these mechanisms, but it is our feeling that a closer study of the *interactions* between these cellular phases and of their eventual susceptibility to hormonal influences might help to clear up some of the problems which are still puzzling us today.

Of course, one must keep in mind the possibility that the real organization of the systems occurs at a higher and more delicate level and that it is irreversibly destroyed by disrupting the cellular structure and perhaps even, for some processes, by isolating the tissue from its natural environment. Indeed, the results which have been obtained so far with various hormones appear to argue in favour of this possibility. However, work with reconstructed systems must first be advanced much more than it has been to date, and must have consistently negative results, before such a view can be finally adopted.

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DISCUSSION

DICKENS: I would like to ask about the fructose diphosphatase in liver, because many of our extracts seemed extremely deficient in it. I presume that is because we dialysed them and lost the magnesium?

DE DUVE: There are several possible explanations. First of all, without Mg it would not be active, and in addition ammonium sulphate has a very bad effect on the enzyme, very little activity remains after the enzyme has been treated with ammonium sulphate.

DICKENS: That's very interesting and would confirm our experiments, because these were ammonium sulphate fractions.

G. CORI: I just want to say how greatly interested I was in Dr. de Duve's analysis of the fate of fructose-1-phosphate. The evidence for the presence of a phosphofructomutase had never been satisfactory.

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Leloir

and his co-workers have also found the co-enzyme of galactowaldenase (uridine-diphosphate-glucose) in liver, and just recently it has been published (*Nature*, London, 169, 1061, 1952) that the enzyme is present in the mammary gland of the lactating rat. Therefore we now know that it is present in one animal tissue.

FOLLEY: Yes. That is the first time that it has been found in animal tissue, I think.

BEST: I'd like to bring the clinicians into this discussion, Dr. de Duve, but I don't know just how to do it. What enzymatic disturbances would you expect in diabetic coma, where the serum potassium is very low?

DE DUVE: I know of only two enzymes in this chart which actually are known to require potassium—fructokinase and pyruvokinase. In addition, as you know, Dr. Hastings *et al.* have shown that the presence of potassium in the incubatory fluid is very important in experiments with liver slices in order to get positive uptake of glucose and synthesis of glycogen. Even the effect of insulin, which they have now described in this system, appears to depend on the amount of potassium present in this fluid (*Fed. Proc.*, 11, 227, 1952).

MIRSKY: Dr. de Duve, have you any studies on the utilization of fructose by muscle?

DE DUVE: Dr. Hers has investigated the breakdown of fructose-1-phosphate in muscle and it goes the same way as in the liver. The

[illegible]

gating the product of the fructokinase reaction in muscle, because *glyceraldehyde should be formed from fructose if fructose-1-phosphate* is the product.

C. F. CORI: In muscle, there is an enzyme that converts fructose-1-phosphate to fructose-diphosphate in the presence of ATP. I think this is confined to the muscle, and the enzyme is not present in other tissues.

DE DUVE: Yes, that is true, but even in the presence of ATP, muscle extract will form glyceraldehyde also when intermixed with fructose-1-phosphate. We have a competing system therefore, and by using an excess of aldolase, one should be able to trap part of the fructose-1-*phosphate, if it is formed.*

THE ENZYMATIC SYNTHESIS AND STRUCTURE OF GLYCOGEN

CARL F. CORI

In this report I wish to describe recent experiments by Drs Illingworth, Larner, G. T Cori and myself which make possible a fairly complete description of the structure of branched polysaccharides of the glycogen-amylopectin class. The method of analysis consisted in step-wise degradation by separate and consecutive action of two enzymes—phosphorylase and amylo-1,6-glucosidase. Step-wise synthesis has also been achieved by separate and consecutive action of phosphorylase and the branching enzyme from liver or muscle.

The two types of bonds that occur in these polysaccharides, the α-1,4 bond in linear portions of the molecule and the α-1,6 bond at branch points, are dealt with by the three enzymes as follows: Phosphorylase makes the 1,4 linked chains longer or shorter by addition or removal of glucose units, and thereby modifies the structure in such a manner that the other two enzymes can act Degradation by phosphorylase exposes the 1,6 linked glucose unit at branch points which can then be split off as free glucose by amylo-1,6-glucosidase. Conversely, when a chain reaches a certain critical length through the synthesizing action of phosphorylase, it can be acted upon by the branching enzyme in such a manner that a 1,6 link is formed from a 1,4 link through transfer of a chain segment.

Consideration of this mechanism of enzymatic synthesis permits certain deductions to be made about structure. It may be recalled first that phosphorylase cannot initiate the formation of a polysaccharide chain from glucose-1-phosphate; it can act only if there are chains present onto which the enzyme can build. This is the so-called primer or starter action, and it has been shown that chains of 4 or more glucose units

can act as nuclei for the synthesis of linear chains of 100 or more glucose units. When there is added to this system a purified fraction of branching enzyme from the liver, the polysaccharide synthesized has the characteristics of glycogen.

The synthesis under these conditions may be represented schematically as shown in Fig. 1, and the question is what kind of structure would result. Two possibilities are illustrated. It

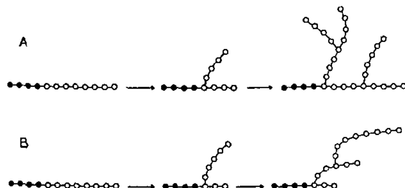


FIG. 1. Schematic representation of combined action of phosphorylase and branching enzyme A—represents the growth of the molecule when all chains have an equal chance of being made longer by phosphorylase In B—only one of the branched chains is made longer by phosphorylase action

is assumed in Fig. 1A that the new end groups created by branching all have the same affinity for phosphorylase, so that there is an equal chance for any of the branched chains to be made longer A continuation of the process shown in Fig 1A would result in the formation of a tree-like structure, such as has been proposed by K. H. Meyer In Fig. 1B it is assumed that one branch has a much higher affinity for the enzyme than the other branches, so that it alone would be made longer, and this would give rise to the laminated structure proposed by Haworth

A distinction between these two possibilities is afforded by the kinetics of the reaction. With low primer concentration,

the curve for polysaccharide synthesis with phosphorylase plus branching enzyme is S-shaped (Fig. 2). The interpretation is that phosphorylase is far from saturated with respect to primer end groups and that the type of branching illustrated in Fig. 1A gives rise to a rapid increase in primer concentra-

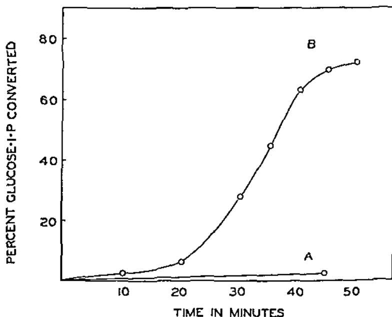


FIG 2. ... accharide. A—phosp ... or phos- phorylase ... B—phos-

tion. On the other hand, formation of a laminated structure would not give an autocatalytic curve, since the number of effective primer end groups remains the same in each molecule that is being built up.

Fig. 3 shows a recent experiment by Dr. Larner in which a chain labelled with ^{14}C glucose units has been used as a means of investigating the mechanism of action of the branching enzyme. The experiment consists of several steps. Rabbit liver

glycogen is first incompletely degraded by phosphorylase (30 per cent out of a possible 36 per cent). This reduces the average length of the outer chains from 9 to about 5 glucose units. The missing glucose units in the outer chains are then replaced by labelled ones, by incubation of the partially degraded polysaccharide with ^{14}C glucose-1-phosphate and phosphorylase. From the amount of glucose-1-phosphate which has reacted, the length of the outer chains can be calcu-

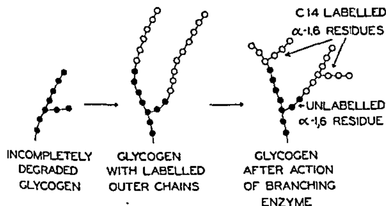


FIG 3 Radioactive labelling technique used to investigate the action of the branching enzyme

lated. In this manner labelled polysaccharides with different lengths of outer chains could be made, in order to find out how long a chain has to be before branching can occur.

The labelled polysaccharides were incubated with a purified and dialysed preparation of branching enzyme from liver or muscle which was free of amylase Whether or not branching has occurred can be detected through the specific action of amylo-1,6-glucosidase which splits off the glucose unit in 1,6 linkage as free glucose For this purpose, the polysaccharide is first digested with phosphorylase in order to expose the glucose unit in 1,6 linkage, so that the glucosidase can act. Before action of the branching enzyme 95 per cent of the radioactivity was found in the glucose-1-phosphate released by

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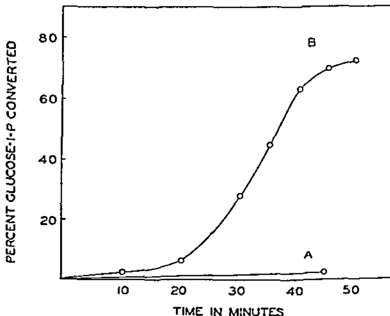


FIG. 2 Conversion of glucose-1-phosphate to polysaccharide. A—phosphorylase alone or branching enzyme alone or phosphorylase + heat-inactivated branching enzyme. B—phosphorylase + branching enzyme.

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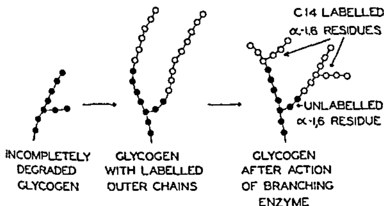


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phosphorylase action and none in the free glucose released by glucosidase action. After branching the free glucose showed a specific activity about one-third of that of glucose-1-phosphate.

This indicates that the branch unit is formed from a unit previously situated in a linear portion of the molecule by a type of transglucosidase action, involving perhaps the shift of a whole chain segment in the exchange of a 1,6 for a 1,4 bond. It has also been found that the outside chains must be at least 8 glucose units long before they can be acted upon by the branching enzyme. This type of branching would explain why the outer branches of glycogen (range 7 to 12 glucose units) are always longer than the inner branches (range 3 to 5 glucose units) As an outside chain grows to a critical length, it can undergo branching with the result that the inner chains formed are shorter than this critical length

Our concept of the enzymatic synthesis of glycogen envisages the simultaneous action of phosphorylase and branching enzyme, and differs somewhat from that developed by Peat and collaborators. These workers believe that amylose—the linear component of starch—is formed first, and is then slowly transformed to amylopectin through the action of Q enzyme. Conversion would be incomplete since starches contain about 25 per cent amylose. There is, however, no amylose present in animal tissues.

We found a much more rapid action of animal branching enzyme on the outer chains of glycogen and amylopectin than on amylose. The action of Q enzyme alone on the outer chains of amylopectin has not been investigated, and it will be of interest to determine what its chain length requirements are. It is known, however, that the kinetics of polysaccharide synthesis by phosphorylase plus branching enzyme in the presence of low primer concentration are the same for the animal and [illegible] to that shown [illegible] ars to be no [illegible] glycogen and amylopectin.

A macromolecule built up by the type of branching shown in Fig. 1A will look as follows (Fig. 4). This is a schematic representation insofar as it is based on a complete regularity of enzyme action; it serves to illustrate certain characteristic features of structure and it permits comparison with the structure actually found by step-wise enzymatic degradation of glycogen and amylopectin

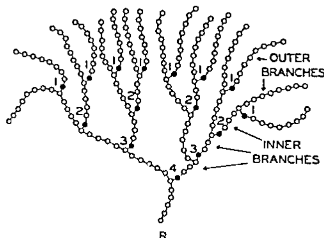


FIG. 4. Schematic representation of structure of glycogen The [illegible]

groups Therefore (16 × 100)/7 7 = 208 glucose residues, or (×162) = M W 33,700 Adding another tier would double the outside branches and the molecular weight

The branch points, indicated by black dots, occur in successive layers or tiers and they become fewer in number as the reducing end is approached The structure shown has four tiers and a molecular weight of 34,000 If five more tiers were added, the molecular weight would be of the order of one million, such as has been found for glycogen by ultracentrifugation or osmotic pressure measurements Irrespective of

the molecular size, the outermost tier would contain 50 per cent, the next 25 per cent, the next 12·5 per cent, and so on, of the total branch points present. In a laminated structure, on the other hand, the percentage of branch points present in each tier would remain the same.

A decision between these two possibilities could be reached by step-wise enzymatic degradation. Experiments have been carried out with liver and muscle glycogen from rabbit and man, and with corn and wheat amylopectin.

The first degradation with crystalline muscle phosphorylase results in the formation of a limit dextrin in which the branch units in 1,6 linkage have been exposed. Phosphorylase can neither break nor by-pass this linkage. The limit dextrin after exhaustive phosphorylase action is isolated and subjected to the action of amylo-1,6-glucosidase in a medium free of inorganic phosphate. When the action of the glucosidase has reached an end point, the branch units in the first tier have been split off as free glucose and this opens the way for the second phosphorylase step which results in the formation of limit dextrin 2, and so on. In some cases 4 successive limit dextrins have been isolated, when the degradation reached 90 per cent. From the analytical values obtained after each enzymatic step, only one kind of model could be constructed, namely one which represents glycogen and amylopectin as multibranched, tree-like structures. The degree of regularity of the structure can be deduced from the per cent branch points present in each tier. In the case of muscle glycogen this was 50 per cent in the outermost tier, 20 per cent in the next, and 10 per cent in the third tier, while a completely regular structure would give 50, 25, and 12·5 per cent respectively. Similar results for the distribution of branch points in successive tiers have been obtained with liver glycogen and with corn and wheat amylopectin.

The glycogens and amylopectins form a continuous series of branched polysaccharides, and they differ mainly in the degree of branching and the length of the inner and outer chains. This statement is based on a survey of glycogens from

ten animal species and from one plant source (sweet Indian corn), as well as of amylopectins from four different starches. The range of values is given below and may be compared with the values obtained with glycogens isolated from 10 cases of von Gierke's disease.

Table I

Polysaccharide	*Per Cent End Group*	*Average Number of Glucose Residues*	
		Outer Branches	*Inner Branches*
Animal glycogen	8·5–5·8	7–12	3–5
Phyto-glycogen	8·4	7	5
Amylopectin	5·9–4·1	13–18	5–6
Glycogen from von Gierke's disease	13·1–4·7	4–15	3–7

The degree of branching (per cent and group) was determined enzymatically as follows: On complete degradation of these polysaccharides with phosphorylase plus amylo-1,6-glucosidase, one obtains the 1,4 linked residues as phosphorylated glucose and the 1,6 linked residues as free glucose, each being determined separately. Hence, (free glucose × 100)/(free + phosphorylated glucose) = per cent end group (since the terminal non-reducing glucose units or "end groups" are related to the branch units as n to [n−1]). It was shown that enzymatic and chemical (methylation or periodate oxidation) end group determinations carried out on the same sample were in good agreement.

The per cent end group varied considerably in the same species, depending on the previous nutritional history and on age. It was 8·1 in glycogen isolated from rabbit liver with low content, 6·8 in a pooled sample from well-fed rabbits and 6·0 in glycogen freshly deposited after a fasting period. The per cent end group decreased (7·7 → 6·9 → 6·5) in glycogens isolated from the livers of embryonal, new-born and young adult guinea pigs. A similar decrease (9·3 → 8·4 → 7·6) was noted in phyto-glycogens isolated from immature, mature and over-mature kernels of sweet maize.

Normal human liver and muscle glycogen fell within the range of values for animal glycogens shown above. In 2 out of 10 cases of von Gierke's disease the glycogen structure was definitely abnormal, one glycogen resembling a phosphorylase limit dextrin and the other amylopectin.

DISCUSSION

RUSSELL: Could you tell us what kinds of glycogens were studied? Were they particulate glycogens or glycogens isolated after alkaline hydrolysis or acid extraction?

C. F. CORI: These were soluble glycogen preparations. The particulate glycogen preparations contain a lot of impurities and they are most insoluble. We have done a few studies with them and they are degraded in the same way as the soluble glycogens. I do not know whether the particulate form is the native state of glycogen or an artifact formed during homogenization. These were all water-soluble polysaccharides, obtained by alkali digestion. Purification consisted in repeated pre- ... solates ... recipi- ... lucose ... pared ...

enzymes. The method of purification of glycogens is therefore of considerable importance in this type of work.

LONG: Was there any difference in the structure, depending on the original source of the glycogen, for example that derived from glucogenic amino-acids, which presumably are a large part of its source during fasting?

C. F. CORI: We have not investigated the structure of glycogen formed from amino-acids, but they all would have to go through glucose in any case and I suppose that the transformation of amino-acids to ...

or does the shorter chain represent the speed with which it has been formed?

C. F. CORI: The length of the outer and inner chains is a distinguishing feature between different polysaccharides, and depends on the relative rates of activity of branching enzyme and phosphorylase. I suppose that liver glycogen in fasted rabbits is highly branched because the branching enzyme has had more time to act on it.

DICKENS: I should like to ask Dr. Cori if he has detected any fructose

[illegible]

quite a considerable percentage, around 5 per cent, and I wondered two things about that: (1) Whether the fœtal glycogens in those species (sheep, cow, goat) which are known to have fructose in the fœtal fluids generally contain much fructose? (2) If there is a specific phosphorylase known which would incorporate fructose?

It seems a very interesting aspect of fœtal metabolism

C. F. CORI. The fœtal glycogen from guinea pig livers could be degraded completely by the two enzymes, if there were any fructose moiety in the molecule, I think it would stop enzymatic action. The crystalline muscle phosphorylase is specific for α-glucose-1-phosphate.

DE DUVE: Dr. Cori, have you any evidence that the reducing ends of the [illegible]

[illegible]

by solutions of glycogen isolated by gentle procedures, but has a pronounced effect when the glycogen has been extracted by the Pfluger method They consider the possibility that alkali-treatment may oxidize some of the reducing groups in the molecule

C. F. CORI We do not know, because we only approached the reducing end by stepwise enzymatic degradation, leaving 10 per cent of the molecule intact As for the light-scattering method for the determination of molecular weights, I wonder whether it is the best method that one can use on a polysaccharide of the size of glycogen, especially when one considers the difficulties of purification, which are not generally appreciated. Impure glycogen solutions are turbid and yet observations in terms of weig[illegible]

to be present I [illegible]

solution, one car[illegible]

hundred million. [illegible]

these polysaccharides has not been completely solved, but I doubt whether the molecular weight is as large as that

YOUNG I would like to ask Dr Cori if he is satisfied with the evidence that the 1–6 links are α, and also if he finds any relationship between the variations in iodine colour among various samples of muscle glycogen and the percentage of branching?

C F. CORI The evidence is based on the isolation in crystalline form of the disaccharide isomaltose, also called brachiose (from brachium = arm), from enzymatic digests of starch, this was done by Montgomery She demonstrated the structure of this disaccharide by methods which

Normal human liver and muscle glycogen fell within the range of values for animal glycogens shown above. In 2 out of 10 cases of von Gierke's disease the glycogen structure was definitely abnormal, one glycogen resembling a phosphorylase limit dextrin and the other amylopectin.

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MIRSKY Would you have said that the amount of change—the high percentage in the fasted animal—represents the more basic glycogen, or does the shorter chain represent the speed with which it has been formed?

PART II

HORMONAL CONTROL OF THE INTERCONVERSION OF CARBOHYDRATE, PROTEIN AND FAT

HORMONAL INFLUENCES IN THE SYNTHESIS OF FAT FROM CARBOHYDRATE

*F. D. W. LUKENS**

AT the outset, it is fitting to place the synthesis of fat from carbohydrate *in the larger frame of which it is a part.* The

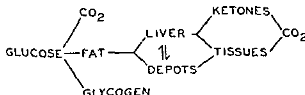


FIG. 1.

conversion of carbohydrate to fat is one of the three major pathways by which carbohydrate is utilized (Fig. 1). The ingenious experiments of Drury (1940) demonstrated the importance of the storage of carbohydrate as fat and the disturbance of this process in diabetes. *Stetten and Boxer (1944a)* were the first to quantitate the principal pathways of carbohydrate metabolism. By the use of deuterium they showed that, of the daily intake of carbohydrate in the rat, about 3 per cent was used for glycogen formation, 30 per cent was converted to fat and the remaining 67 per cent was oxidized.

*From the George S. Cox Medical Research Institute, University of Pennsylvania, Philadelphia, Pa.

length. Starting with short linear chains as primers (too short to give an iodine colour), one can make the chains longer at will by means of phosphorylase, calculate how long they are and then correlate the iodine colour with the chain length. Variation in the iodine colour of

iodine colour.

DE DUVE. In regard to the discovery of β-glucose-1-phosphate formed from maltose (Fitting and Doudoroff, *Fed. Proc.* 11, 212, 1952), does that have to influence our ideas on the structure of glycogen? Have you any comment to make on that?

C. F. CORI. This is different—it is a bacterial enzyme which forms an α 1,4 link from the β form of glucose-1-phosphate Animal and plant phosphorylases, on the other hand, are specific for the α form of glucose-1-phosphate and do not act at all upon the β form Incidentally, *phosphorylase is competitively inhibited by α glucose and not by β* [illegible] the split

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tissues and in cell-free homogenates or enzyme systems. These various combinations of chemical and biological material have been used to gain some information about the part played by the principal metabolic hormones. From this large body of experiment which has been recently reviewed (Gurin and Crandall, 1951) a few examples will be selected. It is hoped

Table I

Principal Variables Involved in the Study of the Synthesis* of Fat from Carbohydrate

Substrate	'*Tissue*'	*Hormone*
	Animal Normal	
	Diabetic	
	Etc	Removal or administration of
Glucose Fructose Acetate Short-chain fatty acids Others	Tissue —Liver Adipose tissue Mammary gland Muscle Etc	Insulin Growth hormone ACTH-cortisone Others
	Cell free enzyme systems	

*Synthesis is often measured by the incorporation of substrates into fatty acids

that they will indicate the present status of the problem of the hormonal regulation of fat synthesis

Most investigators have examined fat synthesis in normal animals and the effect of insulin deficiency on this process. Without discussing the chemical reactions involved in the transformation of carbohydrate to fat, a transformation which appears to require the conversion to a 2-carbon intermediate, the over-all results may be summarized under the following headings

Fat Synthesis in Normal and Diabetic Animals Studied by means of Isotopes

Table II contains some of the data reported by Stetten and Boxer (1944*b*) and by Feller, Strisower and Chaikoff (1950) In the results of Stetten and Boxer (1944*a*, *b*), the figure for glycogen formation in normal rats amounted to 3 per cent of

This largest item, oxidation, is calculated by difference in their work but has since been confirmed by direct measurement in studies with ^{14}C glucose. The exact proportion of each pathway would of course differ with different dietary conditions In the animals of Stetten and Boxer (1944*a*), which were at *constant weight, the calories expended were derived from* glycogen, fat and directly oxidized glucose in corresponding proportions. Whether carbohydrate is converted to fat for storage or as a step towards ultimate oxidation or for both simultaneously, appears to depend on experimental conditions Certainly the formation of fat is the most apparent link between what is called carbohydrate metabolism and the equally important field of fat metabolism. One may assume that when fat has been formed from carbohydrate it then *becomes part of the scheme of fat metabolism and* is subject to the laws governing the transport, storage and oxidation of fat. *The* mobilization of fat, ketogenesis, and *the formation* of cholesterol and phospholipids will not be discussed.

The relation between the fields of carbohydrate and fat metabolism, which is not yet clearly defined, becomes particularly complex when one thinks of the part played by hormones in regulating these metabolic processes. One must face the possibility that hormones may influence the synthesis [illegible] directly, by acting on some part [illegible] by altering other pathways of [illegible] (*c*) indirectly by affecting the [illegible] variety of ways. I shall try to *outline certain studies of the more or less direct action of the* hormones on the synthesis of fatty acids from carbohydrate precursors *Whether Dr Wilhelmi later completes the discussion of this,* or whether he outlines the hormonal influences outside of this limited topic, *he will have plenty to do.*

The magnitude of the problem may also be seen by an *abbreviated statement,* in general terms, of the methods used Table I shows that a variety of substrates have been examined for their capacity to form long-chain fatty acids. The different substrates have been tested in intact animals, in isolated

glucose oxidation in the diabetic rat has been observed (Stetten *et al.*, 1951). The impairment of fat synthesis was not directly measured in these experiments. For the most direct and striking demonstration of the effect of insulin deficiency on fat synthesis, one must turn to the studies on isolated tissues. The liver has been most intensively studied

Table III

Carbohydrate Metabolism of Dogs

	Glucose oxidized	*Body pool*	*Turnover time*	*Glucose turnover accounted for by*		
				Oxidation	*Excretion*	*Undetermined*
	g per hr	*g per dog*	*hrs*	*per cent*	*per cent*	*per cent*
Normal	1 7–2 3	3–4	1 2–1 7	78 96	0	4–22
Depancreatized	0 6–0 8	12–21	4–5	18–21	43 53	29–38

From Feller *et al* (1951)

The Metabolism of Various Carbohydrates by Liver Slices from Normal, Diabetic and Insulin-Treated Rats

Table IV shows that the phenomenon of "starvation diabetes" is the result not only of decreased oxidation of glucose but also of the virtual abolition of fat synthesis from glucose (Masoro *et al*, 1950). It is important for investigators to realize that certain processes, notably fat synthesis, can be reduced to the diabetic level by fasting, and that conditions under which carbohydrate is normally well utilized must be employed to demonstrate the significance of insulin deficiency. Table IV also shows the great increase in both oxidation of glucose and fat formation when normal or diabetic rats are pre-treated with insulin.

The effects of experimental diabetes on the conversion of various substrates to fatty acids by the liver are also shown (Table IV) In this table, the methods used in the study of acetate differed from those used for isotopic glucose or fructose so that the absolute values for acetate cannot be compared with those for the hexoses. The figures for normal fed animals

dietary carbohydrate and was not altered in diabetes. Due to the lability of glycogen, the size of the glycogen pathway may well be somewhat larger than this, but the general significance would not be altered. One may note that the formation of 1·9 g. of body fat from glucose means that 5 g. of glucose or more was used in this way, accounting for 30 per cent of dietary glucose (Stetten and Boxer, 1944a). This figure might

Table II

CARBOHYDRATE METABOLISM OF RATS STUDIED BY MEANS OF ISOTOPES

Rats	Glucose			Body Fat from Dietary Glucose	Dietary Glucose to Glycogen
	Oxidized	Excreted	Unaccounted		
	mg per 100 g per hour			g per day	g per day
	A Studies with deuterium (1)				
Normal		0		1 9	0 44
Diabetic		140		0 1	0 41
	B Studies with ^{14}C glucose (2)				
Normal	69	0	34		
Diabetic	59	80–135	19–78		

(1) From Stetten and Boxer, 1944a, b
(2) From Feller, Strisower and Chaikoff, 1950

well explain the "unaccounted" glucose in the normal rats studied with ^{14}C-glucose (Feller, Strisower and Chaikoff, 1950). The excretion of glucose by diabetic rats was about the same in both investigations. In the normal rats, the proportion of glucose oxidized was 67 per cent. This figure was estimated by difference and hence is not tabulated in the experiments with deuterium It was measured as $^{14}CO_2$ in the expired air when isotopic glucose was used. In the diabetic rats studied with labelled glucose, the slight reduction in the oxidation of glucose may be due to the persistence of some insulin after alloxan. In any case the studies summarized in Table III on the fate of ^{14}C-glucose in the depancreatized dog by Feller, et al. (1951) show that, whether one looks at the grams per hour or at the percentage of glucose oxidized, glucose oxidation is reduced to a third or a fifth that of the normal animal in the absence of insulin. Recently, a similar reduction of

Table V

CONVERSION OF RADIOACTIVE ACETATE, ACETALDEHYDE AND OCTANOATE TO LONG CHAIN FATTY ACIDS BY LIVER SLICES FROM NORMAL AND DIABETIC RATS

Exp No	*Blood glucose mg/100 ml*	*Substrate*		*Insulin*	*Recovered long chain fatty acids*			
		Formula	*Radioactivity administered counts/min*		*Mg per 2.5 g liver slices*	*Radioactivity*		*Micromoles substrate incorporated*
						counts/min	*per cent*	
1	Normal	$^{14}CH_3COONa$	17,000	−	55	5,800	34	25
	,,	,,	,,	+	50	8,500	50	37
2	,,	$^{14}CH_3{}^{14}COONa$	21,000		40	3,200	15	17
	,,	$^{14}CH_3{}^{14}CHO$	,,	−	40	6,000	29	33
	,,	,,	,,	+	40	8,700	41	48
3	,,	$^{14}CH_3COONa$	56,000	+	75	8,600	15	37
	,,	$CH_3(CH_2)_6{}^{14}COONa$	19,000	+	77	1,200	6	9
4	450	$^{14}CH_3COONa$	17,000	−	150	350	2	1.5
	,,	,,	,,	+	135	310	2	1.4
5	420	$^{14}CH_3{}^{14}COOH$	21,000	+	130	390	2	2
	,,	$^{14}CH_3{}^{14}CHO$	,,	+	132	530	3	3
6	650	$^{14}CH_3COONa$	5,600	+	45	51	0.9	0.7
	,,	$CH_3(CH_2)_6{}^{14}COONa$	6,700	+	50	11	0.2	0.2

All the experiments designated by the same number were performed on aliquots of pooled slices

From Brady and Gurin, 1950b

in the first line of Table IV provide a control and a contrast to the impaired oxidation of glucose and the practical cessation of fat synthesis in the alloxan diabetic rats. On the other hand, both fructose and acetate are oxidized to the normal degree by the diabetic liver slice. Nevertheless, in

Table IV

CARBOHYDRATE METABOLISM OF LIVER SLICES IN MEDIA CONTAINING ^{14}C GLUCOSE, FRUCTOSE OR ACETATE

Type of rat	*^{14}C isotope in medium*	*Per cent of added ^{14}C per g liver recovered in*	
		CO_2	*Fatty acids*
Normal (1)			
Fed	glucose	3 1–4 4	0 5–1 3
Fasted 24 hrs	,,	2 5–5 0	0 04–0 07
Fasted 48-72 hrs	,,	0 6–2 3	0 00–0 04
Diabetic (alloxan)– fed (2, 3)	glucose	0 9–1 9	0 00–0 02
Diabetic + insulin	,,	4 6–9 2	1 9–5 0
Normal + insulin	,,	12 2–14 5	6 20–8 14
Diabetic (4)	glucose	0 5–1 9	0 00–0 05
,,	fructose	4 8–7 0	0 02–0 22
Normal	,,	5 4–7 6	0 5–0 98
Normal (4, 5)	acetate	23–37	6–14
Diabetic (4, 6)	,,	17–37	0 6–0 8

(1) Masoro *et al*, 1950 (2) Chernick *et al*, 1950
(3) Chernick and Chaikoff, 1950 (4) Chernick and Chaikoff, 1951
(5) Brady and Gurin 1950*a* (6) Brady and Gurin, 1950*b*

diabetes, fat synthesis from fructose or acetate is as seriously impaired as that from glucose. This impairment of a major pathway of utilization may well explain why fructose has failed to serve as a practical substitute for glucose in diabetic patients in spite of some evidence of its better utilization (Miller *et al.*, 1952)

In Table V, these observations are extended. First, some of the results obtained by the addition of insulin to the medium are shown. In the normal animals this insulin effect on fatty acid synthesis from acetate is significant. In slices from diabetic animals it is not demonstrable, in contrast to the effect

as one might expect. (2) On the other hand, during the increased fat synthesis in the adrenalectomized rats, "the amount of body fat was nearly constant during the period of observation." From this they conclude that the increased synthesis is accompanied by an increased utilization of fat This means that "the adrenalectomized animal diverts a greater proportion of carbohydrate through the metabolic

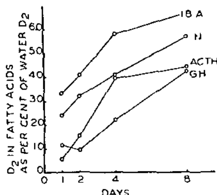


FIG. 2 The rate of increase of carcass fatty acids measured by

pathway of fat synthesis and combustion" (Welt and Wilhelmi, 1950).

Brady, Lukens and Gurin (1951) pre-treated rats with cortisone and found a marked inhibition of fat synthesis from labelled acetate by the liver slices, as shown in the first four experiments of Table VI. This agrees with the response to ACTH observed by Welt and Wilhelmi. Since both inhibitors of fat synthesis, growth hormone and ACTH, have been removed by hypophysectomy, it is not surprising to see in Table VI, experiments 5 and 6, that the amount of fat synthesis by the livers of hypophysectomized rats is 2 to 3 times that for

of pre-treatment of the diabetic animal seen in Table IV. This provides an *in vitro* example of the insulin resistance of severe diabetes which requires a period of exposure to insulin before it is overcome. Such a period is not possible under *in vitro* conditions. Without stopping for detailed discussion of the chemistry, it may be stated that both acetate carbons were equally well incorporated into fatty acids. The inclusion of another substrate, octanoate, shows that in the case of short-chain fatty acids, insulin is also required for their conversion to the long-chain acids, probably because a breakdown to acetate, or an equivalent 2-carbon fragment, is the pathway by which this is accomplished (Gurin and Crandall, 1951).

The Effects of Pituitary and Adrenal Hormones on Fat Synthesis

Having seen that the administration of insulin has an accelerating action on fat synthesis, the effect of the principal contra-insulin hormones, growth and adrenocorticotrophic hormone, may be examined. The first study of these hormones on fat synthesis was that of Welt and Wilhelmi (1950), who used the uptake of deuterium in the liver and body fat of rats to measure the effect of the hormones The reader is referred to the original presentation for experimental details and for a meticulous statistical analysis of the results These may be illustrated by Fig. 2 which has been prepared from their data. This summarizes the results with carcass fat, and liver fat followed a similar pattern. The curves show the rate of incorporation of deuterium into fatty acids during a period of eight days. After noting the normal rate of fat synthesis it can be seen that this is strikingly increased after adrenalectomy and retarded or inhibited when normal rats were treated with either growth hormone or ACTH. All differences from the normal curve were statistically significant. Fig. 2 does not show two things: (1) Treatment with growth hormone caused a progressive decline in the amount of carcass fatty acids similar to that described by previous workers (Lee and Ayres, 1936). Impaired fat synthesis and loss of body fat go together

Table VII

Conversion of Radioactive Acetate to Fatty Acids by Cat Liver Slices

No. of Animals	Condition	Administered Radioactivity		Recovered Radioactivity		Moles Acetate Incorporated/100 mg F.A.
		C/M/MgC	Total	C/M/MgC	Total	
6	Normal	10,000	18,000	7–20	350–1500	2 0–8 0
3	Pancreatectomized (8–50 days, Post Op.)	9,600	17,000	0–2	0–88	0–0 7
7	Houssay (4–43 days, Post-Op.)	10,000	18,000	5–41	100–1000	1 8–9 3
2	Houssay + Growth Hormone	10 000	18,000	0–3	0–120	0–0 7
5	Adrenalectomized – Pancreatectomized (3 11 days Post Op.)	10,000	18,000	1–18	83–080	0 3–5 0

From Brady, Lukens and Gurin 1951

the controls in these experiments. Even with this large synthesis there is an insulin effect.

Table VI
EFFECT OF INJECTED CORTISONE OR HYPOPHYSECTOMY ON THE CONVERSION OF LABELLED ACETATE TO FATTY ACIDS BY RAT LIVER SLICES

Exp No	*Treatment*	*Days*	*Insulin*	*Micromoles substrate incorporated per 100 mg recovered fatty acids*
1	Normal		–	34
	Cortisone 10 mg	3	–	9
2	Normal		–	28
	Cortisone 10 mg	4	–	10
3	Normal		–	31
	Cortisone 20 mg	3	–	6
4	Normal		–	23
	Cortisone 5 mg	3	–	12
5*	Normal		–	14
	Normal		+	26
	Hypophysectomized		–	39
	Hypophysectomized		+	46
6*	Normal		–	9
	Normal		+	12
	Hypophysectomized		–	31
	Hypophysectomized		+	39

*In these experiments rats of the Sprague Dawley strain were used In all others, rats of the Wistar strain were employed
From Brady, Lukens and Gurin, 1951

Experiments with Doubly Operated Animals

In view of the effects of insulin and of the anti-insulin hormones described above, it seemed appropriate to examine the relation of these hormones to each other by means of doubly operated animals. For this purpose hypophysectomized-depancreatized (Houssay) cats and adrenalectomized-depancreatized cats were prepared Normal and depancreatized cats were used to complete the series of experiments which Table VII summarizes. The depancreatized cats had received no insulin for 48 hours; the doubly operated animals had had no insulin since the second operation (pancreatectomy), i.e. for the post-operative days given as ranges in the table. The synthesis of fatty acids from acetate by the normal cat agrees

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DISCUSSION

FOLLEY I think you said that you didn't get direct addition of acetate to short-chain fatty acids, and that you thought that they had to be broken down to 2-carbon units before you got the build-up of long-chain fatty acids?

LUKENS. Well, that is the general working hypophysis which is held today, namely that the channel for synthesis is through some 2-carbon compound

FOLLEY Popják and his collaborators have recently demonstrated very nicely the mechanism of the formation of long-chain fatty acids in mammary tissue They showed that it does take place by the successive addition of acetate units, or the active 2-carbon units derived from acetate, to the carboxyl end I would have thought, therefore, that you might have expected to get elongation of short fatty acid chains.

LUKENS I am aware of the fact that the addition of 2-carbon radicals to short chain fatty acids has been demonstrated I don't know how large a part this plays in mammary tissue but I believe that in the liver it is a minor component of the total fat synthesis

FOLLEY Another point which interested me was that you said that Gurin has found that liver slices from diabetic rats showed no response to insulin

LUKENS That was true for liver slices in these experiments

FOLLEY As you know, Stadie has been studying the mechanism in isolated tissues, and he postulates that insulin combines with active groups on the cell surface I think that I am right in saying that when he pre-incubates tissues which are sensitive to insulin as regards effects on glycogenesis, he only needs to pre-incubate them for a few minutes and then, if he washes the insulin away, he still has the enhanced effect on glycogenesis.

LUKENS I know about Stadie's work. The effect of insulin *in vitro* in these experiments occurred in the normal animal but not in the diabetic under the conditions used That does not mean that one might not get an insulin effect under different conditions

MIRSKY What was the dosage required?

LUKENS Insulin was used in a concentration of 1 unit per ml

with that found in the rat. Fat synthesis is almost abolished by pancreatectomy, as in the studies with diabetic rats and dogs. When the pituitary or adrenals were also removed (Houssay and adrenalectomized-depancreatized animals) the synthesis of fat was normal or nearly so. The two Houssay animals treated with growth hormone (Armour) showed striking inhibition of fat synthesis. From these experiments it appears that fat synthesis is extremely dependent on hormonal regulation. Since, under these circumstances, fat can be formed in the absence of insulin, the part played by the contra-insulin hormones is emphasized.

In conclusion, it has been amply demonstrated that insulin is essential to normal fat synthesis and that fat formation is strikingly impaired in experimental diabetes. The anti-insulin hormones, growth, ACTH and cortisone, inhibit fat synthesis and are probably responsible for the conditions found in severe diabetes. The quantitative importance of fat synthesis and of its regulation in diabetes is apparent, particularly as a consequence of the work of the last 8 years. Finally, these results, based on the most direct methods of observing fat synthesis, must be viewed in relation to other effects of these hormones on carbohydrate and fat metabolism if we are ever to comprehend the full significance of the metabolic hormones.

The advice of Professor Samuel Gurin in the preparation of this manuscript is gratefully acknowledged

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[illegible]

that there are two different enzymes involved for the utilization of glucose and fructose. The initial steps are catalysed by different enzymes.

KOLLER I would like to ask Dr. Lukens how he explains the well-known observation that patients taking cortisone, as well as patients with Cushing's disease, increase their fat deposits?

LUKENS: Well, I do not know how that takes place exactly—or inexactly, even In the presence of a great deal of cortisone it may be possible to have some inhibition of fat formation, which still permits a slow accumulation of fat. The cortisone-treated patient cannot be quantitatively compared with the patient with Cushing's syndrome.

MIRSKY: In the relatively uncompensated patient with diabetes with demonstratable ketosis, cortisone produces a cessation of the ketogenesis. I think that will fit in with what Dr. Wilhelmi suggested, that thereby you may have a retention of fat rather than breakdown.

LUKENS: If one gives cortisone to the Houssay animal, there is just as much fatty change in the liver as in an untreated diabetic animal. This is a gross change.

BEST: It might be actual inhibition of the function of the cells which caused it.

LUKENS: But the mobilization of fat from depots is not the same as an inhibition of lipogenesis.

CAMPBELL In the untreated diabetic cats, I suppose that if you do not give them insulin they do get fatty livers as in the dog?

LUKENS: Yes.

CAMPBELL. But not in the Houssay cat?

LUKENS Yes, but only to a very slight degree.

MIRSKY The point which I'm trying to get at is that with glucose uptake, a marked effect occurs with 0 02 unit/ml., whereas to affect lipogenesis we have to use a minimum of 0 1 unit per ml. I think that the marked differences in effects depend on dosage.

FOLLEY I shall show in my paper that we get threshold effects on lipogenesis in the mammary gland with 0 1 μg. insulin/ml

SHERLOCK If I may, I would like to bring this on to a lower plane and try to get some clinical advice. We heard this afternoon that diabetic animals had difficulty in converting carbohydrate to fat We have also learnt that growth hormone and cortisone administration also result in difficulty in converting carbohydrate to fat How can one correlate that with the clinical types of diabetes? Young diabetics are usually thin and have little fat in the depots or in the livers, yet they are sensitive to insulin On the other hand, the elderly ones are obese, have much hepatic fat, and presumably convert a lot of carbohydrate to fat, yet are insulin-insensitive Excess of growth or adrenal cortical hormone is often postulated in this group. Perhaps Dr Lukens could tell us how this compares with the animal world?

LUKENS No, I certainly cannot I might say that the test of insulin sensitivity is superimposed upon a set of metabolic conditions which might or might not be found at the time of a particular test dose

LAWRENCE If a practising diabetician is allowed to make a comment, and I suppose the problem is linked with the human disease, I don't see why you should expect the insulin to show an effect in the older fat diabetics, because they already have plenty of their own. The addition of a little more is not needed and will not make much difference That is a possibility

LUKENS Dr Stadie's work in which the inhibition of the uptake of insulin by the diaphragm by various hormones is being demonstrated, gives laboratory confirmation of the point you make

LAWRENCE I am not sure, Dr Lukens, that I had time to absorb your slides, but it seemed to me that the giving of fructose which you showed, although non-lipogenic, greatly increased the utilization of carbohydrate. Is that correct?

LUKENS That is correct, under those in vitro conditions

LAWRENCE Well, then, how does that work? I mean, I have assumed that fructose goes into glucose in the liver; how is it so well metabolized as fructose per se?

LUKENS There is a very rapid and immediate effect upon the addition of fructose to a medium. When Dr Max Miller tested fructose in patients, there was a very slight elevation of oxidation for a short period of time, but then its conversion to glucose by the liver, which Dr. Cori mentioned, comes into play and there is no significant difference between fructose and glucose after that conversion takes place. We may be exaggerating a phenomenon by the use of an in vitro mechanism.

LAWRENCE Then if you give fructose to an animal without a liver, what happens to it?

C. F. CORI I think that the answer to Dr. Lawrence's question is

to fat and subsequent oxidation. Animals of this kind may be particularly well suited for the study of lipogenesis.

The extreme instances of fasting and of single-meal training represent exaggerations of the phases of the metabolism of normal animals with dietary habits of regular but intermittent feeding, who alternate between periods of surplus and storage and periods of living off the stores. It is by now clear that the ability of normal animals to make the necessary adjustments in metabolism depends mainly on a balance between the secretions of the pancreatic islets, the pituitary, and the adrenal cortex. Our knowledge of the contributions of the individual hormones to these metabolic adjustments is at present incomplete and somewhat disorderly, but for the purpose of a brief and probably too simple summary, these actions may be considered under two headings. those tending toward the expenditure and those tending toward the conservation of carbohydrate.

The actions of insulin seem to fall entirely under the first heading. It is reasonably well established that insulin facilitates the uptake of glucose by the cells of nearly all tissues and makes possible its rapid entry into the system of phosphorylated intermediates through which glucose is stored as glycogen, is oxidized, or converted into fat (for review, see Krahl, 1951). Whatever the exact nature of this action of insulin may be, it does not seem to be directly related to the effect of insulin on lipogenesis, for three main reasons. (*i*) Insulin promotes lipogenesis from acetate as well as from glucose, and this would appear to require another point of action than that at which it promotes the uptake of glucose. (*ii*) Although an active process of gluconeogenesis is continuously in play in the diabetic animal, this intracellular glucose production, presumably involving the intermediates of the Embden-Meyerhof-Cori cycle, does not lead to fat synthesis in the absence of insulin (*iii*) Although liver slices from diabetic animals can assimilate and oxidize fructose at normal rates (Chernick, Chaikoff and Abraham, 1951) lipogenesis from fructose does not take place readily unless insulin is

THE RÔLE OF THE ANTERIOR PITUITARY IN *THE SYNTHESIS OF FAT FROM CARBOHYDRATE*

ALFRED E. WILHELMI

In his presentation of the main points of evidence bearing upon the hormonal regulation of lipogenesis from carbohydrate, Dr. Lukens has indicated that one of our present tasks is to fit the evidence that he has presented into the framework of intermediary metabolism and into the general pattern of the actions of the metabolic hormones. The object of this paper will be to present, in a summary and tentative fashion, a picture of the relations between the pituitary, the adrenal cortex, and the pancreas, which it is hoped can provide a more coherent and unified view of the actions of these glands on carbohydrate and lipid metabolism

Evidence for the importance and magnitude of the process of lipogenesis as a pathway of carbohydrate utilization has already been presented. In addition, it may be emphasized that lipogenesis is a *continuing* process in the metabolism of animals maintaining constant body weight and composition on diets which contain normal proportions of the major foodstuffs (Schoenheimer, 1942). Also, the extent of this continuing process may be greatly modified not only by changes in the composition of the diet but also by alterations in dietary habit The marked depression of lipogenesis in fasting has already been commented upon by Dr Lukens. An acceleration of this process has been noted (indirectly, as judged by the rise in R.Q. after a carbohydrate meal, and by the increase in saturation of the fatty acids of liver slices incubated with glucose) in rats trained to eat their daily food requirement in one or two hours (Dickerson, Tepperman and Long, 1943). The trained rat, by necessity, utilizes a greater than normal proportion of its dietary carbohydrate by the pathway of conversion

might be related to the inhibition of lipogenesis from carbohydrate. For this reason one can guess that the action of growth hormone may be primary in this respect, and that it may be intensified, or only fully realized, at certain levels of adrenal cortex activity. To answer the question properly, however, one needs to know (*a*) whether growth hormone can inhibit lipogenesis in the adrenalectomized animal, and (*b*) whether cortisone alone can inhibit lipogenesis in the hypophysectomized animal. Neither of these critical experiments has as yet been done

The carbohydrate-conserving actions of the pituitary and the adrenal cortex are also seen, indirectly, in the rapid loss of liver and muscle glycogen in fasting hypophysectomized animals and in the rapid loss of liver glycogen in fasting adrenalectomized animals. The loss of muscle glycogen in the fasting hypophysectomized animal may be prevented by injecting purified growth hormone during the fast (Russell and Wilhelmi, 1950) So far as the loss of liver glycogen is concerned, the limited rate of gluconeogenesis in both hypophysectomized and adrenalectomized animals may certainly play a part. The evidence already presented, that lipogenesis is accelerated in both adrenalectomized and hypophysectomized animals, leads one to wonder whether some of the loss of liver glycogen might not be due to the absence of the restraint upon lipogenesis which is seen to develop in the livers of normal fasting animals. It would therefore be of great interest to know something of the extent of lipogenesis from glucose and acetate in liver tissue from *fasting* adrenalectomized or hypophysectomized animals So far as the author is aware, such experiments have not yet been done.

The carbohydrate-conserving actions of growth hormone and ACTH have their concomitants in the effects of these hormones on the catabolism of fatty acids. Both growth hormone and ACTH have been shown to be ketogenic in normal fasting rats (Bennett, Kreiss, Li and Evans, 1948, Bondy and Wilhelmi, 1950), and growth hormone has been found to increase ketone body production by liver slices from

given or unless the animals have been fed a diet of 58 per cent fructose for several days before the experiment (Baker, Chaikoff and Schusdek, 1952). The argument of these authors that this may be due to the flooding of the organism with 3- and 2-carbon fragments derived from the metabolizable sugar is not very strong, since the active continuing process of gluconeogenesis in diabetic animals would seem to imply an ample stock of such intermediates.

The pituitary and the adrenal cortex seem to be concerned mainly with carbohydrate conservation. As we have seen, growth hormone and ACTH or cortisone, exert a marked inhibition upon lipogenesis from both carbohydrate and acetate (Welt and Wilhelmi, 1950, Brady, Lukens and Gurin, 1951). Since this pathway of carbohydrate metabolism is so important quantitatively, this action of growth hormone and cortisone may easily be the most important of their several effects on metabolism. One point of great interest and importance remains to be settled: are the two hormones independent or related in their action on lipogenesis? If they are related, it is essential to know which is primary in its effect and which is supporting or "permissive." An instance of the latter type of relation is seen in the action of growth hormone in depressing the R.Q. and increasing the storage of muscle glycogen in animals given a carbohydrate meal. This effect is not obtained in adrenalectomized animals unless, along with the growth hormone, a small amount of cortisone (ineffective by itself) is also given (Russell, 1938, Milman and Russell, 1950; Illingworth and Russell, 1951). Another instance is the observation (Engel, Viau, Coggins and Lynn, Jr., 1952) that growth hormone can induce glycosuria in normal rats, force-fed with a high carbohydrate diet, if ACTH is also given. At a constant dose of growth hormone, the glycosuria is about the same when the dose of ACTH is varied over ten-fold, but at a constant dose of ACTH, the glycosuria varies directly with the dose of growth hormone. It will be seen that depression of the R.Q., increased glycogen storage, and spilling of excess sugar are all actions reflecting a carbohydrate-conserving tendency that

from acetate is increased or decreased, lipogenesis from glucose is affected in like manner. The first postulate, therefore, is that the assimilation of acetate into fatty acids is coupled in an obligatory manner with the utilization of an intermediate derived from carbohydrate. Secondly, since both glucose and fructose are lipogenic, the critical intermediate must be common to the two sugars. The synthesis of fatty acids is therefore

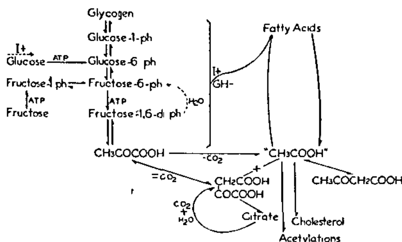


FIG 1 Explanation in text

operated by a "carbohydrate-coupled feedback" of acetate (which may be derived from fatty acids themselves, by beta-oxidation, or from glucose via pyruvate in the usual manner) The third postulate is that insulin facilitates the formation of the critical intermediate from glucose and promotes its utilization in the feedback Finally, the fourth postulate is that growth hormone inhibits the utilization of carbohydrate in the feedback and that its action in this respect may be reinforced by adrenal cortical hormone.

These relations are outlined schematically in Fig. 1. It will be noted that insulin is required for the primary step of glucose utilization, but not for the assimilation of fructose. In liver,

fed normal rats, or from hypophysectomized rats also treated with cortisone (Tepperman and Tepperman, 1951). Both growth hormone and cortisone, acting jointly, appear also to be concerned with the mobilization of fat to the liver in fasting mice (Levin and Farber, 1952). The two latter effects may also be instances of the "permissive" action of the adrenal cortex on the effects of growth hormone.

In thinking, therefore, of the rôle of growth hormone and ACTH in fat synthesis from carbohydrate, it is necessary to take into consideration the other actions of these hormones on glycogen storage, carbohydrate utilization, ketone body production and fat mobilization, and to relate these actions to the effects of insulin on these aspects of metabolism. The first important step in the development of a unifying concept of these relations was taken by Brady and Gurin. They noted that although lipogenesis from acetate and lower fatty acids such as octanoate is almost completely suppressed in liver slices from diabetic animals, acetate (or acetoacetate) production from octanoate proceeds at normal rates, and acetate oxidation appears to be little different from normal. Their *simple and interesting explanation of these observations* is that the process of acetate production from fatty acids by beta-oxidation is not directly reversible, but that the incorporation of acetate into fatty acids occurs by a different metabolic pathway. In the absence of insulin, the latter pathway is blocked and lipogenesis ceases, while fatty acid oxidation and acetate and acetoacetate production may continue unimpaired (Brady and Gurin, 1950). Their idea is illustrated in the right hand side of Fig. 1. In their view, the catabolism and anabolism of fatty acids represent two phases of a cyclic process, the synthesizing half of which is under the influence of insulin. *The restoration of lipogenesis in the Houssay animal* also implies that this process is influenced in addition by the *pituitary* (Brady, Lukens and Gurin, 1951).

This attractive hypothesis may be modified to cover a great deal more ground by adding to it a few simple postulates. First, it is noted that in every instance in which lipogenesis

ketone body production, and increase liver glycogen or hepatic glucose output. With both ACTH and cortisone, effects of both kinds have been observed (Engel, 1952).

In the diabetic animal, the feedback may be at least partly blocked by the unopposed action of growth hormone and the adrenal cortex. Removal of the pituitary relieves this inhibition, and carbohydrate utilization by this pathway may be resumed at its natural or unregulated rate. The feedback operates to a degree, and nett fatty acid catabolism and ketone body production are diminished. Such a preparation is, however, unstable and sensitive to the carbohydrate supply to insulin and the growth hormone.

If we may assume that the metabolic organization of the liver is somewhat specialized but not fundamentally different from that of other tissues, this hypothesis might be applied to them as well. In muscle, for instance, the action of growth hormone in diminishing lipogenesis from carbohydrate could lead to the observed phenomena of depression of the R.Q. and increased storage of muscle glycogen after a carbohydrate meal. It may be worth emphasizing that in this instance insulin and growth hormone are not entirely antagonistic, since glucose uptake is nót inhibited. In fact, it has been observed that the administration of insulin to glucose-fed rats treated with crude anterior pituitary extract has little effect in raising the R Q. but does bring about spectacular increases in muscle glycogen (Russell, 1938) One other interesting consequence of this hypothesis as applied to muscle is that the rate of ketone body utilization may also be determined in part by the extent to which the feedback is in operation in the peripheral tissues.

A detailed account of the suggested consequences of the scheme would take far too much time and space, and it is hoped that many more aspects can be brought out in the discussion. There are two points that may be made in conclusion. First, the essence of the hypothesis is due to the excellent work and the bold suggestion of Brady and Gurin; its faults in extension and in its probable oversimplification of these complex relations

the pathways of the two sugars converge at fructose-6-phosphate; in muscle, fructose-1-phosphate appears to be phosphorylated to yield fructose-1, 6-diphosphate (Cori, Ochoa, Slein and Cori, 1951) At the moment there is not enough evidence to indicate the nature of the common intermediate that is coupled with lipogenesis. The point of convergence of carbohydrate and fatty acid catabolism is in the two-carbon unit indicated as acetate. From this point acetate may be assimilated into the feedback, it may be oxidized, after condensation with oxaloacetate, via the citric acid cycle, it may be utilized for the synthesis of cholesterol or it may be employed in the acetylation of foreign amines, and finally, if it is produced in excess of the capacity of other systems to consume it, acetate may be condensed with itself to form acetoacetate.

A few of the consequences of this scheme may be briefly outlined. In the fasting normal animal the depletion of liver glycogen leads to a reduction in the amount of carbohydrate available to operate the feedback. Acetate assimilation by this pathway is reduced, and the nett breakdown of fatty acids is increased. There may be a small increase in ketone body production. As the process of gluconeogenesis from protein accelerates, the flow of carbon tends to be from rather than towards the citric acid cycle, so that acetate utilization by this pathway may also be diminished. If in these circumstances hormone is administered, the diminished feedback may be inhibited, resulting in a greater nett breakdown of fatty acids and an increase in ketone body production, the "ketogenic" effect.

In the light of this scheme, ACTH or cortisone might be expected to have two different effects, depending on the circumstances. If insulin is present and growth hormone activity is relatively low, then the action of cortisone in promoting gluconeogenesis may be sufficient to increase the supply of sugar to the feedback, decrease the nett catabolism of fatty acids, and reduce ketone body production If, however, insulin activity is low and growth hormone activity is relatively high, cortisone may reinforce the inhibition of the feedback, increase

glycogen effect, it has been shown that cortisone and allied compounds do influence the carbohydrate metabolism of hypophysectomized rats Lukens and Dodds, as well as ourselves, have shown that cortisone aggravates the diabetes of the Houssay animal, so that there is at least one example of the independence of the growth hormone effect from the presumably ACTH cortisone effect. This question as to the independence or interdependence of the action of these two hormones will I think come up time and time again here A good deal of stress will be laid, and has been laid, on observations of the deficient state of the adrenalectomized animal or the animal treated with growth hormone I think it well to remember the opposite circumstances, that when a large amount of cortical hormone is given to the normal animal, the effects are positive and are presumably independent of any other pituitary hormone

WILHELMI But the normal animal has its own pituitary, and you may be attaining a level at which certain aspects of the pituitary action are expressed, whereas at low levels of adrenal cortical activity one may not really see the full effects of the depression of pituitary hormones.

LONG I think it is well to remember though, that you do have a cortisone effect in hypophysectomized animals—at least on their carbohydrate metabolism

RUSSELL About the effect of cortisone in hypophysectomized animals, in the experiment to which Dr Long referred the increased deposition of carbohydrate was less than that in intact or adrenalectomized animals given the same amount of cortical extract, and small in proportion to the effect on nitrogen excretion It appeared that the usual effect of the extract on glucogenesis had been obtained, but not the interference with carbohydrate utilization which may be seen in the animal with a pituitary

LONG That's perfectly true, Dr Russell In fact, while the stimulation of glucogenesis as judged by nitrogen excretion is just as large in the hypophysectomized animal given cortical extract, the accumulation of liver glycogen is not so great It would appear that in the hypophysectomized animal there is another deficiency operating against the accumulation of the liver glycogen found in the adrenalectomized or intact animal

WILHELMI In this connection, it might be worth while to emphasize that if you calculate on a carbon to carbon basis, the potential carbohydrate that can be derived from protein is actually much greater than is ordinarily realized in diabetic animals Therefore, if there is an

LONG That is true, because in our calculations we used the factor 3 65 to 1, but obviously there is still a considerable proportion of carbon to be accounted for

must be borne by the author. Secondly, the hypothesis does not account for the most important relation between insulin and growth hormone. their combined action in nitrogen retention and growth. It does lead to the speculation, however, that the action of growth hormone in suppressing carbohydrate utilization for fat synthesis may be related to the requirement of carbohydrate in some special form for protein synthesis.

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DISCUSSION

LONG. Dr. Wilhelmi posed a question as to the cortisone effect in hypophysectomized rats, he remarked that the actual experiments had not been carried out However, so far as the effects on carbohydrate metabolism are concerned, and presumably they are linked with the

C. F. CORI: Would you expect the same mechanism to operate in the incorporation of glycine into glutathione?

WILHELMI. I think it is different. One possible extension of this idea is that one could argue that there may be a coupling between carbohydrate utilization and the assimilation of amino acids. One of the ... ydrate utilization by fatty ... the required synthesis of ... ot too far—I didn't want ... unresolved state—but I admit that this is a pure speculation so far as the coupling goes, the CHO in the inhibited feed-back system is conserved for other purposes, one of which may be the support of protein synthesis

BEST Is this "feed-back" conception primarily yours, Dr. Wilhelmi, or Gurin's?

WILHELMI Well, I think it really belongs to Brady and Gurin They made the initial suggestion that the assimilation of the acetate was probably by a different pathway from the breakdown of the fatty acids to C_4 units They did not, however, make the assumption that this was coupled in any obligatory fashion with carbohydrate utilization.

LUKENS I hope that Dr Wilhelmi will some day include an historical reference to the obligatory utilization of CHO, we never knew what we were talking about in the old days—and I am not sure whether we do now!

G CORI Perhaps one should remember that fructose is converted to glucose in the liver, and that is the reason why fructose is not a satis-

take place in the gastro-intestinal tract, as well as in the liver Thus, if fructose is fed, it is even less efficient than when it is administered intravenously

YOUNG I would like to emphasize the question of dietary factors in the ætiology of ketogenesis, and to recall some experiments which Marks and I did about fifteen years ago, in which we showed that the

which is capable of inducing ketonuria on administration to a normal rat on a high fat diet What the mechanism is, I don't know It is a substance present in the lipid fraction of meat, which appears to be capable of catalysing the formation of ketone bodies from fat in the

ketonuria. Indeed Petren says that he gave 100–200 gm of butter fat

WILHELMI: What did your calculations show, Dr. Russell?

RUSSELL: 8·25 was the value for protein of average composition. This is the theoretical maximum D:N if all the carbons are converted to glucose, or spare equivalent glucose carbons from oxidation

LUKENS: In connection with these experiments with cortisone in hypophysectomized animals, we did several experiments giving cortisone to Houssay animals which were not included in the fat synthesis work However, there was no doubt that cortisone produces a severe fatty liver in the animal with terminal acidosis. It is a very powerful factor in the Houssay animal.

BEST In regard to "feed-back," I don't like the term very much and don't understand why it is used Perhaps this is due to defects in my knowledge—it is an electrical analogy, isn't it?

WILHELMI Yes, it arises from electrotonics—a feed-back circuit is one which puts a little bit of power back to regulate its source It probably is not quite correct to use it in this sense because the scope and mechanism of the process are rather different. I used the analogy to indicate that the return of 2-carbon units to the higher fatty acid level involved a coupling of the whole process with glucose utilization, and that in the absence of that coupling or in restraint, such a return of half of the cycle is inhibited

BEST Perhaps we could have some better term for it—I have no

glycine and measured the incorporation of glycine into the protein

from fasted animals is very low in glycogen

When he repeated these experiments with liver slices from diabetic animals, he found that the incorporation of the labelled glycine into glutathione was very much depressed When he added glucose to the medium, this restored somewhat the incorporation into glutathione.

I don't know what the feed-back mechanism is, but it must be explained in terms of some intermediary reaction. What is the chemical or enzymatic basis for the feed-back mechanism, that is my question?

WILHELMI: I can't answer that question at the moment because

EFFECTS OF INSULIN AND CORTICOIDS ON LIPOGENESIS *IN VITRO*

S. J. FOLLEY

THAT lipogenesis is an important route of carbohydrate disposal in animals has been known from the time of the classical experiments of Lawes and Gilbert. In view of the crucial rôle of insulin in carbohydrate metabolism it might be expected that this hormone would be concerned in the regulation of the conversion of carbohydrate to fat. Indeed such a rôle for insulin has been postulated on the basis of experiments on the whole animal (Drury, 1940, Stetten, Jr., and Boxer, 1944).

The question of the effects of insulin and other hormones, added *in vitro*, on the metabolism of isolated tissues has received increasing attention in recent years, the majority of the earlier studies being concerned with glycogen synthesis and breakdown. Bloch and Kramer (1948) were the first to report a stimulating effect of insulin *in vitro* on lipogenesis in tissue slices (rat liver) but their findings could not be confirmed in all breeds of rat studied (Bloch, 1948) More convincing evidence of the stimulation of lipogenesis in rat liver slices by insulin added to the incubation medium was later presented by Brady and Gurin (1950).

Our own studies on this question have been carried out on lactating mammary gland slices, mammary tissue being, as we have pointed out (Folley and French, 1949, 1950), a tissue especially suitable for studying lipogenesis *in vitro*, and we have used manometric and isotope incorporation methods for this purpose. Our manometric measurements have been of two types first, determinations of the so-called composite respiration curve, which is essentially the curve of overall pressure changes resulting from the metabolism of tissue slices

to bring his patients out of diabetic coma. I don't see how that observation fits in with anything which we hold at the present time about the intermediary metabolism of fatty acids, but certainly our own results agree with Petren's observations.

BEST From what meat was this crystalline substance isolated?

YOUNG We got it from horse muscle, and also from calf intestinal mucosa

BULLOUGH We've heard a lot this afternoon about the inhibitions caused by growth hormone—it inhibits hexokinase, it inhibits fat synthesis, it depresses the R.Q. To this I can add that it inhibits epidermal cell division *in vitro* How does it come about that it is also a growth hormone?

WILHELMI I can't answer that. As a matter of fact, if you take the data from human responses to the injection of growth hormone, this cannot be regarded as growth hormone Most of the emphasis and references in the literature are to the responses of animals other than man to our present preparations of growth hormone.

CONN I have some data that I plan to show later this afternoon dealing with the effects of the Raben-Westermeyer growth preparation in a human being We were able to demonstrate definite nitrogen retention in man without any evidence of diabetogenic activity This human subject sh
effect since he was
diabetes mellitus.

BEST. I want t
(Salter and Best)

CONN If I may add something to what Dr Best has just said—in the patient that I've just described we were able to show marked nitrogen retention by administration of insulin alone.

Folley, 1951). These results have since been confirmed by Hills and Stadie (1952).

Similar though somewhat smaller effects were obtained with glucose as substrate but insulin had no noticeable effect on the metabolism of rat mammary gland slices incubated with acetate alone (Table I). In the latter connection it should be noted that rat mammary tissue incubated with acetate alone

Table I

EFFECT OF INSULIN *in vitro* ON THE RESPIRATORY METABOLISM OF MAMMARY GLAND SLICES FROM LACTATING RATS

(*Results from Balmain and Folley*, 1951)

No of rats	*Stage of lactation days*	*Addn of insulin*	$-Q_{O_2}$	*R Q*	Q_{acid}	$-Q_{glucose}$
	Substrate: *acetate* (0 02M)+*glucose* (0 3%)					
10	13–15	+	11 7±0 6	2 07±0 03	−2 5±0 2	9 2±0 9
		−	8 6±0 6	1 53±0 05	−0 7±0 2	4 7±0 8
	Substrate *glucose* (0 3%)					
10	14–15	+	11 0±0 7	1 87±0 04	3 2±0 2	
		−	9 4±0 6	1 55±0 04	3 3±0 3	
	Substrate, *acetate* (0 02M)					
10	12–18	+	4 6±0 4	0 82±0 04	−0 5±0 2	
		−	4 6±0 3	0 76±0 03	−0 2±0 1	

does not oxidize this substrate nor utilize it for lipogenesis as judged by respiratory and isotope incorporation data (Folley and French, 1950, Balmain, Folley and Glascock, 1952*a*).

In unpublished experiments in our laboratory, Miss J. H. Balmain has studied the dose/response relationships of this interesting effect of insulin on lipogenesis *in vitro* Using as response the effect on the slope of the composite curve, she has found that the threshold dose of insulin is in the neighbourhood of 0 002 i.u./ml and that there is a graded increase in response with increasing insulin concentrations up to 0·055 i.u /ml. With Mr. C P. Cox we are at present investigating the possibility of utilizing this response for the assay of insulin.

The above-mentioned increases in $-Q_{O_2}$, R.Q., $-Q_{acid}$, and $-Q_{glucose}$ due to insulin were interpreted as indicating that

incubated with suitable substrates (in the present work, acetate + glucose) in Krebs-Henseleit bicarbonate-saline in equilibrium with O_2-CO_2 gas mixture, and secondly, measurements of R.Q., -Q_{O_2} and -Q_{acid}, *supplemented in some cases* by chemical determinations of -$Q_{glucose}$. Our more recent work has involved, besides manometric observations, measurements of the incorporation of isotopically labelled substrate carbon into the fatty acids isolated from the slices at the end of the incubation period.

Stimulation by Insulin of Lipogenesis from Small Molecules

Fig. 1 shows the effect of insulin (0·05 i.u./ml. of medium) on the composite respiration curve of lactating rat mammary gland slices metabolizing acetate + glucose as substrates. This effect was first described by Balmain, French and Folley (1950) It will be seen that in the absence of added insulin, the overall pressure progressively increases, giving a composite *respiration curve of positive slope. This is undoubtedly due to* the fact that, under these conditions, mammary gland slices from lactating rats exhibit R.Q. > 1, which has been interpreted (Folley and French, 1950) as evidence that the tissue is effecting the net synthesis of fat from small molecules. In the presence of insulin the slope of the composite respiration curve was markedly increased, from which we inferred that insulin increases the R.Q. of the tissue and hence the rate of net fat synthesis.

Actual measurements of R.Q. show that the quotient is indeed increased by addition of insulin to the incubation medium. Table I, taken from Balmain and Folley (1951) shows that insulin (1 i.u./ml.) increased the R.Q. of lactating rat mammary gland slices incubated with acetate+glucose from a mean value of 1·53 to a mean value of 2·07, with a concomitant increase in -Q_{O_2}. At the same time there was an increase in -Q_{acid}, showing that the acetate uptake is increased by insulin, and measurements of -$Q_{glucose}$ indicate that the same applies to the glucose utilization (Balmain and

In experiments on mammary gland slices from lactating rats (Balmain, Folley and Glascock, 1952*b*), we have found that addition of cortisone to the medium decreases the positive slope of the composite respiration curve which is characteristic of the tissue at this functional stage (Fig. 1). This indi-

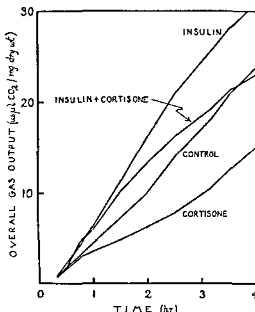


FIG 1. Effects of insulin and cortisone on the gas exchange of lactating rat mammary gland slices incubated in acetate (0 02M)+glucose (0 3 per cent) (from Balmain, Folley and Glascock, 1952*b*)

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cation that, under these conditions, cortisone decreases the R.Q of the tissue and hence the rate of lipogenesis was confirmed in the first instance by measurements of R.Q. and more definitely by experiments with labelled substrates which showed that the incorporation of both glucose and acetate carbon into the fatty acids was decreased by cortisone (Table III). When both insulin and cortisone were present

insulin increases the rate of utilization of both acetate and *glucose carbon for fatty acid synthesis by the tissue. This* conclusion has been confirmed by experiments involving the incubation of mammary gland slices from lactating rats with labelled substrates. At first we used mixtures of [*carboxy*-^{14}C]-acetate + unlabelled glucose (Balmain, Folley and Glascock, 1952*a*) and in later unpublished experiments, doubly labelled acetate [1-^{13}C-2-tritio] acetate + ^{14}C-glucose. A typical example from the latter series of experiments (Table II) shows that insulin increases the incorporation of both the

Table II

LIPOGENESIS IN MAMMARY GLAND SLICES FROM A LACTATING RAT

(Balmain, Folley and Glascock unpublished work)

Substrate ^{14}C-glucose (0 3 per cent) + $CT_3{}^{13}COONa$ (0 02M)
^{14}C 10 μc /l T 10 μc /l ^{13}C 5 atom per cent excess

	Isotope content of Ca salts of mixed fatty acids after 3 hr		
	^{14}C c p m /mg C	*T c p m /mg combustion H_2O*	*^{13}C atom % excess*
Control	381	95	0 054
Insulin (1 i u /ml)	805	202	0 14

carbons *of acetate and of glucose carbon into the fatty acids* by lactating mammary tissue from the rat

Effect of Corticoids on Lipogenesis from Small Molecules

In view of the antagonism between insulin and corticoids as regards diabetogenesis in the intact animal it was of interest to study the effect of 11-oxygenated adrenal cortex steroids on lipogenesis by mammary gland slices *in vitro*, particularly since Brady, Lukens and Gurin (1951) have shown that pre-treatment of rats with cortisone decreases the incorporation of [*carboxy*-^{14}C]-acetate into the fatty acids of slices of their livers *in vitro*.

energy from the oxidation of acetate alone, glucose nevertheless stimulates the incorporation of acetate carbon into the fatty acids of mammary gland slices from ruminants (sheep), as well as enabling mammary tissue from non-ruminants (rat) to use acetate carbon for lipogenesis (Folley and French, 1950, Balmain, Folley and Glascock, 1952*a*). However, udder slices from lactating ewes, when incubated with labelled acetate + labelled glucose, utilize much less glucose carbon and more acetate carbon for lipogenesis than do rat mammary slices. Results of a typical experiment (Balmain, Folley and Glascock unpublished) illustrating this species difference are given in Table IV

Table IV

LIPOGENESIS IN LACTATING MAMMARY GLAND SLICES IN ABSENCE OF INSULIN
(*Balmain, Folley and Glascock unpublished work*)

Substrate $CT_3{}^{13}COONa$ (0·02M) + ^{14}C-glucose (0·3 per cent)
^{14}C 10 μc/l T 10 μc/l (rat), 20 μc/l (sheep)
^{13}C 5 atom per cent excess

	Isotope content of Ca salts of mixed fatty acids after 3 hr		
	^{14}C *c p m /mg C*	T *c p m /mg combustion H_2O*	^{13}C *atom % excess*
Rat	381	95	0·054
Sheep	22·5	1002	0·373

A further difference between lactating mammary tissue from ruminants and non-ruminants is revealed in the response to insulin *in vitro*. We have found that addition of insulin to the medium is totally without effect on the respiratory metabolism (Balmain and Folley, 1951) or on the incorporation of glucose and acetate carbon into fatty acids by udder slices from lactating sheep (Balmain, Folley and Glascock, 1952*a*; and further unpublished work) Table V shows results of a typical experiment in which sheep udder slices were incubated with $CT_3{}^{13}COONa$ + ^{14}C-glucose.

If it should prove that species differences of this type apply to tissues other than the mammary gland, one would

Table III

EFFECTS OF INSULIN AND CORTISONE *in vitro* ON LIPOGENESIS IN MAMMARY GLAND SLICES FROM A LACTATING RAT

(*Results from Balmain, Folley and Glascock, 1952b*)

Substrate ^{14}C-glucose (0 3 per cent) + CT_3COONa (0 02M)
^{14}C 10 μc/l. T 50 μc /l.

Addition	*Isotope content of Ca salts of mixed fatty acids after 3 hr*			
	^{14}C		*T*	
	c p m /mg C	*% of control*	*c p m /mg combustion H_2O*	*% of control*
None	365	100	283	100
Cortisone	254	70	77	27
Insulin	*795*	*218*	*430*	*152*
Insulin + cortisone	383	105	118	42

together there was evidence of antagonism, since the potentiating effect of insulin on lipogenesis was neutralized wholly or in part.

We felt it of interest to study the dose/response relationships of the effect of glucocorticoids on lipogenesis *in vitro* and, since preliminary experiments by the composite curve technique indicated that corticosterone was appreciably more active than cortisone in this respect, we have used corticosterone in these investigations Our preliminary studies (Balmain, Folley and Glascock: unpublished) so far indicate that relatively low doses of corticosterone (10–50 μg./ml) instead of inhibiting, actually stimulate—and to quite a considerable degree—the utilization of glucose and acetate carbon for lipogenesis, while higher doses exert an inhibiting effect, as found in our previous studies with relatively high concentrations of cortisone. These studies are being continued.

Species Differences in Response of Mammary Tissue to Insulin *in vitro*

Though, as respiration studies (Folley and French, 1949, 1950) show, mammary tissue from ruminants can obtain its

on lipogenesis is bound up with the effect of the hormone on the utilization of carbohydrate for energy production.

However, recent work in our laboratory suggests another possibility for consideration. It should be pointed out that the mammary gland not only builds up fatty acids but incorporates them into glycerides. It is therefore to be expected that in such a tissue the supply of glycerol might well be a rate-limiting factor in fatty acid synthesis from small molecules. Evidence that this is so has come from manometric measurements (Balmain and Folley, 1951) and isotope incorporation studies (Balmain, Folley and Glascock, 1952*a*) which showed that glycerol added to the incubation medium exerts a marked stimulating effect on lipogenesis by rat mammary tissue similar to that of insulin. Table VI shows the results of a typical experiment This raises the question whether the

Table VI

COMPARISON OF THE EFFECTS OF INSULIN AND GLYCEROL ON LIPOGENESIS IN MAMMARY GLAND SLICES FROM A LACTATING RAT

(Results from Balmain, Folley and Glascock, 1952a)

Substrate $CH_3{}^{14}COONa$ (0 02M) + glucose (0 3 per cent)
^{14}C 50 μc /l

	^{14}C *content of Ca salts of mixed fatty acids after 3 hr*
	c p m /mg C
Control	5951
Insulin (1 i u /ml)	9061
Glycerol (0 2 per cent)	10282

effect of insulin on lipogenesis may be mediated through the breakdown of glucose to glycerol Glycerol could arise as a result of a side reaction at the triosephosphate stage of glucose breakdown, and it is worth noting that recent results indicate that glucose is the main precursor of the glyceride glycerol of the milk and that the conversion takes place in the mammary gland itself (French and Popják, 1951, Popják, Glascock and Folley, 1952)

expect insulin to be less important in the intermediary metabolism of ruminants than in that of carnivores. Were this the case, the fact that pancreatectomy is less severe in its

Table V

LIPOGENESIS IN UDDER SLICES FROM A LACTATING EWE

(*Balmain, Folley and Glascock unpublished work*)

Substrate: ^{14}C-glucose (0.3 per cent) + $CT_3{}^{13}COONa$ (0.02M)
^{14}C 10 μc./l. T 20 μc./l. ^{13}C 5 atom per cent excess.

	Isotope content of Ca salts of mixed fatty acids after 3 hr		
	^{14}C c.p.m./mg C	*T c.p.m./mg combustion H_2O*	*^{13}C atom % excess*
Control	22.5	1002	0.373
Insulin (1 i.u./ml.)	25.3	970	0.334

effects in herbivorous animals than carnivores (see Lukens, 1944) might find some explanation.

Mechanism of the Stimulation of Lipogenesis by Insulin

As pointed out above, the stimulating effect of insulin on lipogenesis in mammary tissue has only been observed in circumstances in which glucose is oxidized and at the same time used as a substrate for lipogenesis, i.e. it has been observed with mammary tissue from non-ruminants (rat) incubated with glucose alone or plus acetate, but not with mammary tissue from ruminants (sheep). Now, as we have seen, mammary tissue from ruminants and non-ruminants respectively appears to differ in the nature of the system which yields energy, since manometric results (Folley and French, 1949, 1950) show that non-ruminant mammary tissue obtains its energy from the oxidation of carbohydrate, while in the case of ruminant mammary tissue acetate is the oxidizable substrate of choice. Since lipogenesis is an energy-requiring process it thus seems possible that the stimulating effect of insulin

FOLLEY: We have done experiments with an alloxan-diabetic goat In these experiments, the animal soon had a very high blood sugar which was controlled by insulin, but the insulin-resistance seemed to

[illegible]

organic metabolism, they are very much less than those produced by cortisone. The major activity of corticosterone in man is upon electrolyte metabolism

We were interested some years ago in the changing electrolyte composition of sweat under the influence of administered deoxycorticosterone or ACTH. Under these circumstances there occurs a sharp decrease in sweat sodium and chloride and an increase of sweat potassium Dr Wolfson tested the effect of corticosterone upon the concentration of electrolytes in human milk and observed the same

[illegible]

[illegible] from acetate in the perfused liver He found

[illegible]

FOLLEY We haven't done many such experiments We have done some experiments involving R.Q determinations but on mammary gland slices only, we got rather indefinite results, so I didn't present them

[illegible]

them.

MANN With reference to Dr Folley's important investigations on the essential difference between carbohydrate metabolism in monogastric animals and in ruminants, I would like to mention some experiments on the mechanism of seminal fructose formation in ruminants and monogastric animals In monogastric animals, such as the rabbits for instance, we can influence the level of fructose in the blood. In alloxan diabetes both go up, and when insulin is injected, both go down In ruminants, the level of glucose in the blood is low Nevertheless, the semen of these animals (e g. ram and bull) has a high fructose

The work discussed in this paper has been carried out in collaboration with the late Dr T H French, Miss J. H. Balmain and Dr. R. F

to the following Dr K Hallas-Moller of Novo Terapeutisk Laboratorium, Copenhagen, for crystalline insulin free from glycogenolytic factor, and to Dr Abraham White of Chemical Specialities Co Inc. for corticosterone

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DISCUSSION

MIRSKY Have you studied the effect of cortisone on lipogenesis?
FOLLEY No. We haven't yet
MIRSKY: The reason I ask this is because of the experiments which show that depancreatized chickens develop hyperglycæmia after the ... It may well be that since insulin does not ... ffect of cortisone on lipo-

... ing point, and we intend ... If you are ... have bred,

RESPONSES OF DOGS TO PURIFIED GROWTH HORMONE*

JAMES CAMPBELL

IN 1945 Li, Evans and Simpson described procedures for the isolation of the growth hormone of the anterior pituitary gland, and Wilhelmi, Fishman and Russell (1948) succeeded in crystallizing the material in good yield by application of methods which were developed by E J Conn and his associates for the separation of plasma proteins These advances have made possible physiological and biochemical studies which reveal that the growth hormone has important effects on metabolic processes, as well as on structures. Investigations now under way are providing links for the correlation of the metabolic with the structural influences. Experimental results that have been obtained indicate that there are probably functional relationships between the pancreas and the hypophysis in regard to the secretion of these hormones.

It has been demonstrated that highly purified growth hormone is diabetogenic in cats (Cotes, Reid and Young, 1949), and in dogs (Campbell, Davidson, Snair and Lei, 1950). Houssay and Anderson (1949) produced permanent diabetes in the partially depancreatized dog by relatively high doses of growth hormone, and Campbell, Davidson and Lei (1950) produced metahypophyseal diabetes in dogs, which previously had been given injections of a diabetogenic pituitary fraction on one or more occasions In the continuation of these studies, the effects produced in dogs by doses of growth hormone adequate to produce diabetes have been determined. These investigations are reported fully by Campbell, Hausler, Munroe and Davidson (1953), and will now be briefly described.

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concentration. How then is seminal fructose formed in the ruminants? We find in the organ which produces fructose in the ruminants, i.e. the seminal vesicle, a very high reserve of glycogen which provides through its metabolism (via phosphoglucose and phosphofructose) the source of fructose.

Another point which I should like to mention is that the spermatozoa both of ruminants and of monogastric animals consume fructose at the same high rate.

analysis, show less than 1 per cent of impurity.* These preparations have very high growth activity and produce intense diabetogenic effects in dogs.

Through fractionation of hog pituitary gland extracts, Raben and Westermeyer (1952) indicate that the diabetogenic factor of the pituitary is distinct from the growth hormone. However, in this laboratory, it has not been possible so far to separate diabetogenic activity from purified bovine growth hormone preparations. To the contrary, in our experience from 1949, the more highly purified growth hormone has given more prompt and intense diabetogenic responses in dogs. If it is postulated that a growth factor could be separated from a diabetogenic factor in these preparations from bovine pituitary glands, the factors must have practically identical molecular weights and have the same electrophoretic mobilities; or alternatively, the hypothetical diabetogenic factor must produce the responses in dogs in doses of less than 5 per cent of those required to produce diabetes, together with 95 per cent of the growth hormone. These possibilities do not appear likely at present

The subcutaneous administration of highly purified growth hormone produced severe diabetes in four previously untreated dogs, in dosage of 3 5 mg per kg. per day (dogs F, P, M and R of body weight 9 2 to 12 kg., Fig 1) The daily diet consisted of 300 g. ground horse meat, 100 g. ground dog chow (Purina) and 40 g. sucrose. Samples of blood were taken in the morning (16 hours post-prandial), then the first injection and the first meal were given, followed after 8 hours by the second injection and meal. A small amount of urine was lost, as exercise was allowed for two short intervals each day Hyperglycæmia, glycosuria, polyuria and polydipsia were elicited in 1 to 2 days of injection and remained as long as the injection continued. The plasma was visibly lipæmic on the second to fourth days of injection, and ketones appeared in the urine. During the diabetes the dogs became noticeably quiet, the

*We are indebted to Dr H D Bett and Mr K A B Degen of the Connaught Medical Research Laboratories for these analyses

The growth hormone was prepared (Campbell and Davidson) from bovine anterior pituitary glands by precipitating the globulin fraction by dialysis and subsequently applying the procedures used by Wilhelmi, Fishman and Russell (1948) for the purification of their Fraction A. The growth activity was determined by the tibia test of Greenspan, Li, Simpson and Evans (1949): doses of 10 and 25 μg. per rat per day for 4 days, in young female hypophysectomized rats, produced increases in the widths of the uncalcified, epiphyseal discs of the tibiæ of 116 and 149 microns over the control values. These responses compare favourably with those obtained with purified growth hormone by Greenspan *et al.* (1949). Since the tests were made in different laboratories the results however may not be compared directly. The preparations contained detectable amounts of other pituitary principles. The ACTH content, assayed by the method of Sayers, Sayers and Woodbury (1948), was estimated to be about 1 per cent of the standard La-1-A. The thyroid-stimulating hormone (TSH) activity, assayed by the method of Ghosh, Woodbury and Sayers (1951), was about 0·10 U.S.P. units per mg. The prolactin activity was of a low order, and was estimated, by the method of Hall (1944), to be equivalent to 5 per cent of the International Standard.

From electrophoretic analyses performed by Dr. A. W. Wilhelmi on another preparation which was used for reference, the main component was identified with the growth hormone preparation of Wilhelmi, Fishman and Russell (1948). Electrophoretic analyses in this laboratory by Dr. E. S. Goranson indicated that the growth hormone preparation used in the present studies contained about 5 per cent of more mobile material. The administration content of the growth hormone preparation determined by Dr. A. F. Graham, agreed with that found for purified bovine growth hormone by Li and Moskowitz (1949). These analyses also showed that about 5 per cent of heavier

mone. On further

have subsequently

in this period and until the end of the experiment. The growth hormone preparation was then administered again to the three dogs in dosage of 2 mg. per kg. per day for 6 to 9 days. Hyperglycæmia, glycosuria, polydipsia and ketonuria again appeared in two of the dogs, on this lower diet and dose, but not in the dog R who was obviously the most resistant.

The diabetes elicited by purified growth hormone corresponds in important respects with that induced previously by crude and partially purified anterior pituitary extracts, and can properly be termed idiohypophyseal diabetes. Since the response can be elicited by a single factor, the growth hor- [illegible]

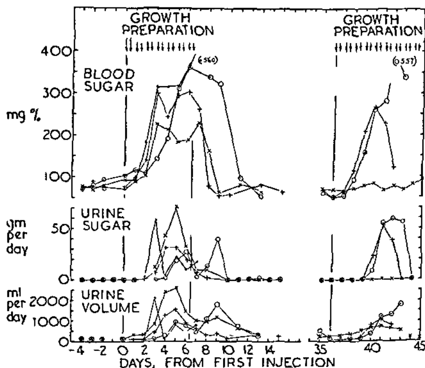
[illegible] the responses to the growth hormone For example, ACTH in relatively high dosage increased the blood and urine sugar of dogs, but to a lesser extent than the growth hormone (Campbell *et al*, 1950). Probably this matter will resolve into a question of semantics.

Examination of the pancreases of these dogs by Dr W. S. Hartroft and Mr W Wilson showed that the growth hormone had produced degranulation of the β cells of the Islets of Langerhans. In the dogs with the most severe diabetes (F and M) the degranulation was practically complete, whereas in the most resistant dog R, granulation was still recognizable in a few of the β cells of the islets, but the amount was much reduced as compared to the normal

The insulin extractable from the pancreases, determined by Dr. G. A Wrenshall, was much less in these dogs given growth hormone than in the five normal control dogs that were given bovine plasma albumin (Fig. 2) In agreement with previous results in dogs given a diabetogenic pituitary fraction (Ham and Haist, 1941), there was good correlation between the degrees of β cell degranulation and the extractable insulin of the pancreas.

The degranulation of the β cells and the reduction of the extractable insulin of the pancreas produced by the growth

appetite decreased and vomiting occurred, while the water intake remained high. The rate of respiration increased and tremors were present in the fore and hind limbs. The signs of diabetes were most intense in dog F, and after 7 days of in-



jection he was found in a state of diabetic coma which proved fatal despite the administration of insulin. A resting period of 30 days ensued for the remaining three dogs, during which the signs of diabetes disappeared in from 2 to 4 days from the last injection. The diet was reduced to half the former amount

hormone is of interest in relation to the blood sugar level. Previously, Best, Campbell and Haist (1939) found that there was in a general sense, an inverse relation between the reduction of the insulin content of the pancreas and the increase in blood sugar in dogs given a diabetogenic pituitary fraction. It was not concluded, however, that the reduction in insulin content depended upon hyperglycæmia. During the first few days of injection in some of these dogs, the insulin content was definitely reduced although the fasting blood sugar did not rise above 124 mg per cent. In the present experiments, although the most resistant dog (R) did not show glycosuria or fasting hyperglycæmia during the second series of injections, marked degranulation of β cells occurred and the insulin of the pancreas was only one-fifth of the normal amount. Hyperglycæmia, therefore, cannot be considered as the sole cause of these effects of growth hormone on the pancreas, nor is it an important cause in the early stages of administration.

Several investigators have studied the effects of hyperglycæmia, produced by continuous parenteral administration of glucose, on the pancreas. Although prolonged infusion of sugar can produce degenerative changes in the pancreatic β cells in cats (Dohan and Lukens, 1948), the β cells pass through different phases in guinea pigs (Woerner, 1938), and blood sugar levels corresponding to those which occur in dogs given diabetogenic pituitary extracts do not decrease granulation, and may even cause islet hyperplasia (Houssay, Foglia, Smyth, Rietti and Houssay, 1942).

It also follows that hyperglycæmia and acidosis can be excluded as principal causative factors for the increases in plasma volume and plasma proteins which result from the administration of growth hormone. It appears, however, that in dogs given growth hormone, the extent of the increase in the fasting blood sugar can be taken as a rough general index of the intensity of the effects of the hormone, but that profound changes can occur in the body fluids and tissues in the absence of obvious changes in blood sugar.

Studies of blood cells and plasma proteins were prompted

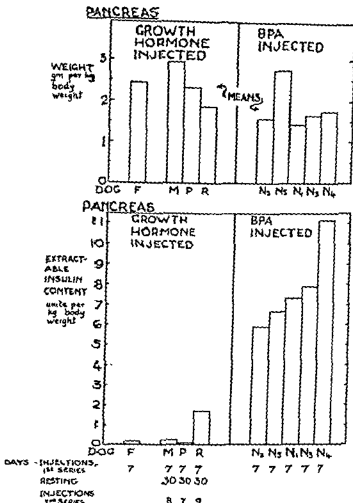


FIG. 2 Growth hormone, D21P, was administered to the test dogs for seven days, in dosage of 3 5 mg per kg of body weight per day The extractable insulin of the pancreas of dog F was then determined After a resting period of 30 days, growth hormone was again given to the other three dogs for seven to nine days, in dosage of 2 mg per kg per day, and the insulin extractable from the pancreas was determined. The control dogs were given 3 5 mg BPA per kg for seven days before extracting the insulin of the pancreas

also produced interesting changes in the plasma proteins. The concentration of total protein in the plasma was increased in the order of about 1 g. per 100 ml. by the administration of the purified growth preparation; as a consequence of this and of the increase in plasma volume, the amount of total protein in the plasma per kg. body weight was increased above the pre-injection values. During the resting period the amount of total protein decreased, and on re-administration of the growth hormone was again increased (Fig 4). It would seem probable that the volume of the plasma may be increased as a result of the increase in the amount of total protein in the plasma.

The concentration of fibrinogen (determined as fibrin) in the plasma was greatly increased by the administration of growth hormone, the values obtained being 2·5 to 8-fold the initial values. Since the plasma volume also increased, the quantity of fibrinogen was much greater than normal (Fig. 4). The increase in fibrinogen is, however, not large enough to account for the over-all increase in the amount of total protein. The difference was due chiefly to increases in the amount of globulins (other than fibrinogen). The concentration of this protein fraction was also increased Thus when the hormone was given, the total protein was increased in amount more by the globulin fraction than by the fibrinogen, but the latter was increased to the greatest extent above the pre-injection levels. The results do not permit of definite conclusions on the effects of growth hormone on the albumin fraction. Although the concentration of this fraction decreased, giving low A/G ratios, the amounts were not decreased, due to the rise in plasma volume. On the occasion of sampling for blood volume determinations, the quantities of albumin found were above the normal during the first series of injections, were still high during the resting period, and were again higher during the second series of injections.

The cause of the increased amounts of protein in the plasma is not known. It is, however, recognized that the growth hormone increases the amount of protein in the body. It

by the observations that in dogs given growth hormone the cells settled out rapidly from blood samples, and that the blood has an increased tendency to clot on withdrawal. It was found (Campbell, Lei and Davidson, 1951) that the erythrocyte sedimentation rate (ESR) was greatly increased by

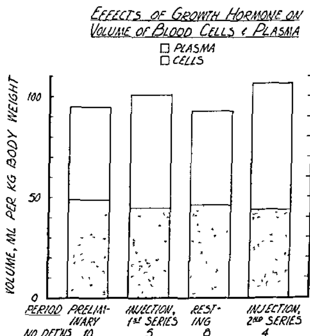


FIG. 3 The average values for four dogs given 3 5 mg growth preparation, D21P, per kg. per day during the first series of injections (seven days), and 2 mg. per kg during the second series (seven to nine days)

the administration of growth hormone and that an increase in plasma fibrinogen was apparently responsible for this effect.

Further investigations have shown (Campbell, Hausler, Munroe and Davidson, 1953) that growth hormone in doses adequate to produce diabetes (Fig. 1) increased the plasma volume per kg. of body weight (Fig. 3). The growth hormone

also decreases the rate of formation of urea in nephrectomized rats. In the dogs given growth hormone, the body weight increased (except in the instance of dog F whose diabetes was so severe as to result in death) and the amounts of nitrogen and of urea excreted were reduced. Since also a slight increase in concentration accompanied the increase in quantity of total plasma protein, it is probable that the rate of synthesis of plasma protein was increased; it is also probable that the rate of utilization of these proteins was reduced. The evidence for increased synthesis is more impressive in the case of fibrinogen, a protein that is produced by the liver (Madden and Whipple, 1940) In the dogs given growth hormone, the fat content of the liver was increased; the liver weight and dry, fat-free solids, which represent chiefly protein, were also increased markedly in relation to the body weight (Fig. 5). The increase in plasma fibrinogen cannot be attributed therefore merely to release of the protein from reserves in the tissues Thus the evidence suggests that the rate of synthesis of this protein by the liver was increased by the growth hormone

The total quantity of cells in the blood per kg. body weight in these dogs was apparently not appreciably influenced by the growth hormone injections (Fig. 3). However, the volumes per cent of packed erythrocytes (Fig. 6), the hæmoglobin concentrations and the red blood cell counts were decreased (Campbell *et al.*, 1953) These effects on cells per unit volume of blood can therefore be attributed to dilution due to the increase in plasma volume

The rapid ESR of the dogs given growth hormone (Fig. 6) can be attributed largely to the effect of the increased concentrations of fibrinogen in causing a tendency towards aggregation of cells. A relatively small part of this effect could be attributed to the reduction in the hæmatocrit readings. Direct observations of the blood flow in the small vessels of the mesentery indicated that in three of the dogs given growth hormone there was an increased tendency towards "sludging" of the blood, as compared to two normal control animals. It is probable that a tendency towards

appears, in the growing animal, that the rate of synthesis of protein is increased and the studies of Young (1945) indicate

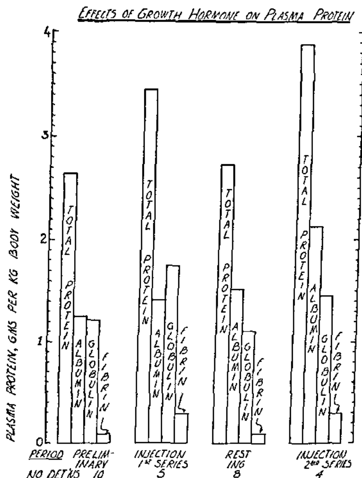


FIG. 4. Notes as in Fig. 2

that the rate of utilization of protein is also decreased by pituitary extracts containing growth hormone. The results of Russell and Cappiello (1949) show that growth hormone increases the rate of uptake of amino acids from the blood and

plasma, determined by the method of Quick (1938), was also decreased (Fig. 7), but the relation of these results to the clotting system is apparently not understood at present (Brambel, 1950). However, these observations may prove to be of in-

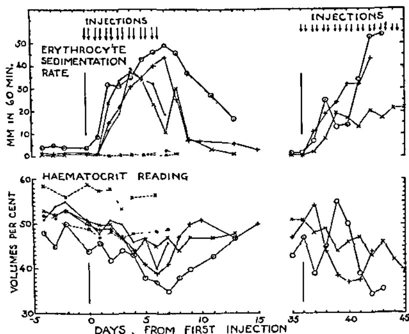


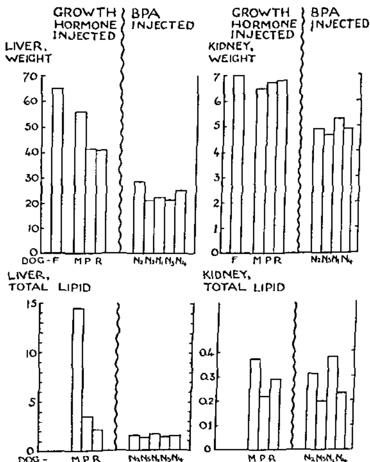
Fig 6 The test dogs were given growth preparation D21P

terest in relation to the considerable increase in weight, and probable increase in the activity of the liver

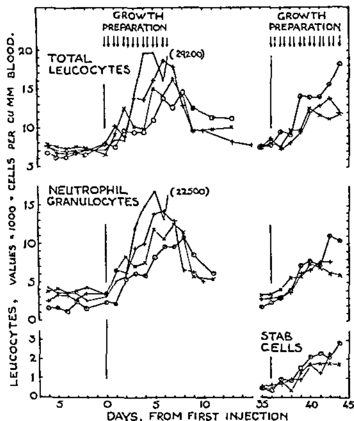
Administration of the growth hormone also produced a considerable leucocytosis in these dogs which proved to be due to increase in neutrophil granulocytes and to a lesser increase in

sludging of blood was promoted by aggregation of cells (Knisely, 1950).

The activity of the clotting system was increased in the dogs given growth hormone, since the clotting time of the blood was decreased (Fig. 7). The prothrombin time of the

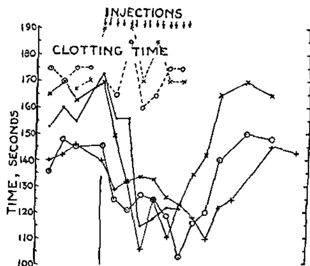


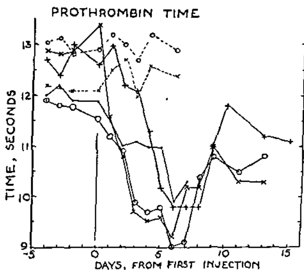
stab cells (Fig. 8). The percentage (per 100 white cells) of lymphocytes, monocytes, eosinophils and basophils decreased,



[illegible]

but the actual amounts of these cells (per c. mm of blood) were not definitely altered. Examination of smears of the bone marrow has not revealed differences in the frequency distribution of the various cell types.





[illegible]—The first dogs F, B, M and P (symbols as in Fig. 6)

between the hypophysis and the pancreas in respect of the secretions of growth hormone and insulin. Recent observations in this laboratory have indicated the possibility that a diminished secretion, or supply, of insulin may produce compensatory hypersecretion of growth hormone. This was suggested by the finding that in depancreatized dogs, maintained on insulin and a diet containing raw pancreas, the ESR and plasma fibrinogen tended to be erratic, and were above normal (Munroe and Campbell). In these dogs, and also in depancreatized dogs given growth hormone, Mr W. Wilson observed deeply staining acidophil cells in the anterior pituitary gland. This observation is suggestive of increased secretory activity of these cells. Analogous relations between the secretion of pituitary hormones and removal of the organs that they influence have been demonstrated. Removal of the thyroid gland, for example, apparently leads to compensatory overactivity of the cells of the anterior pituitary which produces thyrotrophin (Purves and Griesbach, 1951), and gonadectomy is also apparently followed by increased secretion of gonadotropin (Halmi, 1952) There is a suggestion in the results, therefore, that the pituitary and the pancreas may be interdependent not only by the supply of growth hormone influencing the secretion of insulin, but also by the supply of insulin influencing the rate of secretion of the growth hormone.

Acknowledgements

I desire to express my gratitude to my colleagues Dr H R Hausler, Mr J S Munroe and Mr I W F Davidson for permission to outline the new studies that are the result of their united efforts. We wish to thank Professor C. H Best for his unfailing support and encouragement in this work

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In depancreatized dogs deprived of insulin, a considerable leucocytosis was found. This change was characterized by a neutrophilia, but with relatively little increase in stab cells (Campbell, Munroe, Hausler and Davidson, 1953). In the dogs given growth hormone, possibly part of the leucocytosis may therefore be due to the effects on the tissues of lack of insulin. Mere lack of insulin, however, does not account for the increase in stab cells, which occurred in response to excess growth hormone.

Leucocytosis has frequently been observed in individuals with diabetes mellitus who have developed acidosis or coma through lack of proper control. On re-establishing control of the diabetes with insulin, the leucocytosis is reduced. According to Allan (1927) the leucocytosis in these cases is not necessarily dependent on the infections which may also be present; it has been suggested that acidosis is the cause. However, in the dogs given growth hormone, the neutrophilia cannot be attributed to acidosis, since this effect occurred in the dog R in which hyperglycæmia, glycosuria and ketonuria were absent during the second series of injections. Also in all dogs, neutrophilia occurred early during the injections of growth hormone, before ketonuria was found.

There is evidence, derived chiefly from studies on nitrogen metabolism, that diabetogenic anterior pituitary extracts may increase the rate of liberation of insulin from the pancreas (Mirsky, 1939; Young, 1945, Gaebler and Galbraith, 1941; Gaebler and Robinson, 1942). This conclusion was stated also by Haist, Campbell and Best (1940). The more recent studies of Milman and Russell (1950); Milman, DeMoor and Lukens (1951); and Campbell, Munroe, Hausler and Davidson (1953) indicate that purified growth hormone may increase the rate of secretion of insulin by the pancreas. It is possible therefore that increased rate of secretion of insulin may be involved in the production of some of these responses of intact dogs to growth hormone.

These results, and consideration of the effects of hypophysectomy, suggest that there may be functional relationships

DISCUSSION

GAARENSTROOM: I was rather surprised to hear that growth hormone produced a comparative increase in weight of the liver and the kidney, because earlier it was only with non-purified growth hormone preparations that one got an increase in the weight of the internal organs. About ten years ago I did some experiments with partly purified growth hormone in hypophysectomized animals and the animals did show considerable growth, but the kidneys and liver didn't grow at all, nor the heart either. I cannot imagine that your preparation was not a purified one?

CAMPBELL: Yes, it was a purified one. May I ask, were these animals rats which you were using?

GAARENSTROOM: Yes.

CAMPBELL: Li and Young and also ourselves have found that, when given highly purified pituitary growth hormone, the rat does not show an increase in kidney or liver weight relative to the body weight. The [illegible]

rationale being that it was the older animal that was more likely to become diabetic than the younger one. However we were quite unable to increase their blood sugar levels; the old animals grew just as though they were lively youngsters.

I am sorry that Dr. Campbell did not hazard some theory as to what it is that brings about islet damage under the influence of growth hormone. I wonder if I could goad him into making some comments on this point by putting forward my own views, which are still that the damage is brought about by an excessive stimulation of the islets to secrete insulin. I think that idea has been in the minds of many of us for many years. I don't see any evidence available at the present time to dispute it and I would be very interested to hear Dr Campbell's views on this rather controversial matter.

CAMPBELL: I think that the growth hormone causes a great increase of insulin secretion from the β cells, and produces the eventual damage to the cells by over-stimulation, and that this increased output of insulin is probably produced by stimulating the extra-hepatic tissue. The growth hormone causes an increased demand for insulin, and in order to meet this demand there may be an increased secretory activity on the part of the islet cells.

YOUNG: Therefore you would expect the blood insulin content to be low after the administration of growth hormone, ultimately?

CAMPBELL: Yes, ultimately.

LONG: There is another effect of growth hormone over and above that on the pancreas. If you want to throw a Houssay animal into a

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treated cats, and administered that to an alloxan-diabetic, adrenalectomized, hypophysectomized rat, as Dr. Campbell found, we found the blood sugar was raised It may well have been that under the influence of growth hormone, a hyperglycæmic substance was liberated from the pancreatic islets simultaneously with the insulin The situation might well be complicated by the α cell glycogenolytic factor.

CONN: In 1945, we reported experiments in which we administered

patients with spontaneous hypoglycæmia due to islet cell tumours. We had expected the blood sugar to rise Instead, much more severe hypoglycæmia occurred until the extract administration was stopped. These patients, before extract administration, were chronically and severely hypoglycæmic due to excessive secretion of insulin Under these conditions it seemed unlikely that administration of pituitary extract so increased the peripheral need for insulin that more severe hyperinsulinism occurred We, therefore, concluded that the extract contained a principle which stimulated directly the insulin-producing cells of the pancreas.

diabetic coma, just give it the purified preparation, and acidosis and coma quickly develop.

A second point which I want to make is that these same preparations given to fasting animals produce hypoglycæmia.

BEST: Sometimes?

LONG Well, in the rat, I should say regularly. It's an immediate effect, and within a few hours you get hypoglycæmia.

BEST Our Dr Sirek does not find this effect in the normal dog.

MANN. May I ask if any of the changes which you observed after ... increased

... id in

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veloping, wouldn't you?

LAWRENCE *I've never come across it All my new patients get* ...

these dogs do

SWYER *Could I revert for a moment to the question of growth* hormone increase and the effect on the islet cells of the pancreas? I'm not sure if I understand the situation correctly but since growth hormone injections produce hyperglycæmia in the dog, it can't be primarily merely a question of excessive outpourings of insulin, surely?

BEST *Not necessarily, the two effects could take place at the same* time

... effect is obviously

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insulin level *The treatment with growth hormone would* ... the processes which would utilize more of the insulin, and thereby lower the blood insulin level and induce further activity on the part of the islet cells On the other hand, we had the idea that possibly there ... growth hormone which

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provided that food intake is kept constant and that the cat does not become "refractory" (insensitive). From this relationship it is usually possible to estimate the potency of a diabetogenic preparation, relative to that of a reference substance, by performing two tests with the reference substance and an intervening test with the "unknown" preparation (Reid, 1951*b*).

The diabetogenic tests now to be reported were performed

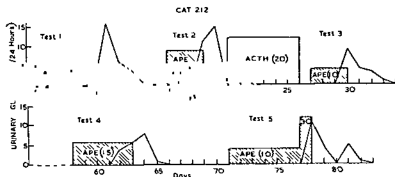


FIG 1 Effect of ACTH on the diabetogenic activity of a crude anterior-pituitary extract (APE)

Period of treatment denoted by rectangle, the height of which is proportional to the daily dose (also indicated numerically). APE (alkaline extract Batch No 297) derived from cattle pituitary glands, dose expressed in ml (1 ml = 0 25 g gland tissue)

ACTH (Armour & Co, Batch no 84-85H) derived from pig pituitary glands, dose expressed as equivalent amount (mg) of La-1-A (Sayers test)

exclusively in cats, in which the pancreas was intact. We have confirmed that the partially-depancreatized rat or alloxan-diabetic rat is highly insensitive to the diabetogenic action of preparations of growth hormone (Reid, 1951*b*). Since the glycosuria of alloxan-diabetic rats is greatly increased by ACTH (Bennett and Li, 1947), and since we have so far been unable to induce diabetes in cats with ACTH, it is evident that the effectiveness of different diabetogenic agents may

ACTH AND GROWTH HORMONE AS DIABETOGENIC FACTORS

E. REID

I WILL not attempt to review the background to the discovery that purified preparations of growth hormone are diabetogenic in test animals such as the cat, as first reported by Cotes, Reid and Young (1949) I propose to consider this finding in some detail, with reference to the possibility that the diabetogenic action of these preparations might be due to a factor distinct from growth hormone itself. I will also discuss the action of ACTH preparations in enhancing the diabetogenic activity of preparations of growth hormone (Reid, 1951*a*).

This action of ACTH is illustrated in Fig. 1, which shows how we test preparations for diabetogenic activity. This cat was given daily subcutaneous injections of a crude extract of cattle pituitary glands, until a diabetic response was obtained, namely the excretion of glucose in excess of 5 g. during 24 hours. With the dose used in this test (No. 1), the response was obtained after 6 days, but with the higher dose used in Test 2 the response occurred after only 3 days. When, however, a low dose was given in Test 3, after the administration of ACTH, the response was again obtained in 3 days. We concluded that the ACTH had rendered the cat hypersensitive to the diabetogenic action of the growth hormone present in the crude extract. When the crude extract was again injected a month later (Test 4), it appeared that the cat was no longer hypersensitive.

From tests 1, 2, 4 and 5 it is evident that there is an inverse relationship between dose and period of treatment required to induce the diabetic response. This dose-response relationship has been found to apply to any cat in our laboratory,

cats, and the growth-promoting potency in rats, of a number of purified specimens of growth hormone, some obtained by Li's method and others by Wilhelmi's method. The data we obtained (Fig. 2) suggest that the ratio of the two activities is

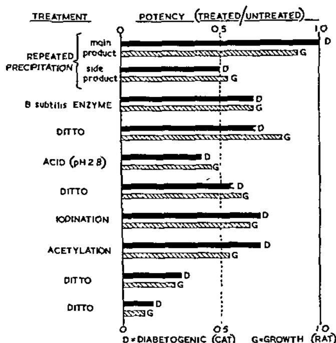


FIG. 3 Effect of certain treatments (cf Reid, 1952a) on the diabetogenic and growth-promoting potencies of purified preparations of growth hormone

In Figs 3, 4 and 7, a potency bar terminating in a "spike" represents a minimum value for the potency, the actual value being possibly higher than that indicated

quite constant, although different preparations may vary in absolute potency A similar broad parallelism between diabetogenic potency and growth-promoting potency was found with specimens which had been partially inactivated by treatments such as acetylation (Fig. 3).

depend on the test animal employed. There is, however, a broad analogy between the finding of Engel, Viau, Coggins and Lynn (1952) and of Ingle and Li (1952) that ACTH can render growth hormone diabetogenic in force-fed rats, and our finding that ACTH can enhance the diabetogenic activity of preparations of growth hormone in cats.

Is Diabetogenic Activity an Intrinsic Property of Growth Hormone?

We have closely investigated the possibility that the diabetogenic activity of growth hormone, as obtained from cattle

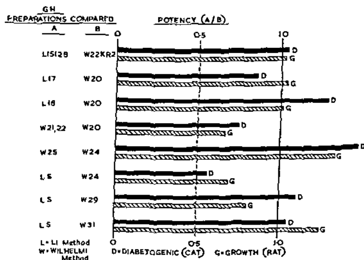


FIG 2 Comparisons of various purified specimens of growth hormone (GH), with respect to diabetogenic and growth-promoting potencies

GH preparations derived from cattle pituitary glands. L1512B and W22KR2 were kindly provided by Prof. C. H Li and Messrs Armour & Co (by courtesy of Prof A. E Wilhelmi) respectively

pituitary glands, might depend not on growth hormone itself, but on some other substance associated with the purified protein and not revealed by the usual physical tests of homogeneity. We have determined the diabetogenic potency in

Raben and Westermeyer (1952) have reported that diabetes could not be induced in dogs with growth hormone derived by a novel procedure from pig pituitary glands. The growth hormone which these authors tested, and which was not diabetogenic even with doses much higher than the effective dose of beef growth hormone, had been treated with oxidized cellulose so as to remove ACTH. Since this treatment was conceivably the key to their apparent success in separating the diabetogenic factor from growth hormone, we have now

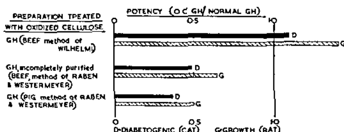


FIG 5 Comparisons of preparations of growth hormone (GH) treated with oxidized cellulose and "normal" purified GH preparations (beef), with respect to diabetogenic and growth-promoting potencies

assayed specimens of growth hormone after treatment with oxidized cellulose.

The diabetogenic potency of such preparations was in no case significantly lower than their growth-promoting potency (Fig. 5). One of the preparations tested was a specimen obtained from pig pituitary glands, and kindly provided by Drs. Raben and Westermeyer by courtesy of Prof. E. B. Astwood. Only preliminary assays (Fig. 5) have been performed with this preparation, but it is already evident that its diabetogenic potency is quite high in relation to its growth-promoting potency, this being only about half that of growth hormone from cattle pituitary glands

The data of Raben and Westermeyer, together with data now obtained, suggest a possible explanation of their failure

An interesting finding which emerged from work with acetylated preparations was that the activity of growth hormone does not depend on the integrity of the α-amino groups which terminate the peptide chains of the protein molecule (Reid, 1952*a*). Experiments with the enzyme carboxypeptidase (Fig. 4; cf. Reid, 1952*b*) have indicated, in agreement with Condliffe and Li (1952), that the amino-acids at the carboxyl

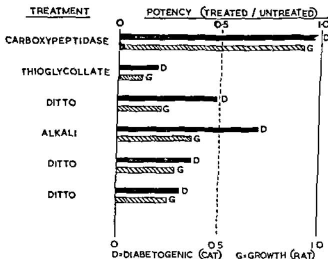


Fig 4 Effect of certain treatments (cf Reid, 1952*b*) on the diabetogenic and growth-promoting potencies of purified preparations of growth hormone

ends of the chains are also dispensable. It is, therefore, possible that the activity of growth ho mone may depend merely on a small portion or constituent of the protein molecule.

Fig. 4 also shows the effect of treating specimens of growth hormone with thioglycollate or alkali. The values for diabetogenic potency were slightly higher than those for growth-promoting potency in the case of these treated preparations, as with the acetylated preparations (Fig. 3), but in no case could the difference be considered significant.

extract of cattle pituitary glands. After some time the sensitivity of the cat fell to the level found in the control test (No. 1) performed at the outset of the experiment. This temporary change in sensitivity is strikingly similar to that occurring after administration of ACTH, as in the experiment shown in Fig. 1. It would however be premature to press this possible analogy, particularly since the action of ACTH preparations

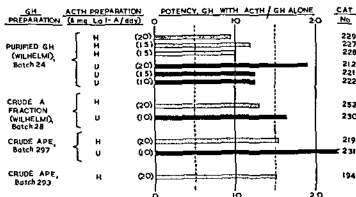


FIG 7. Preliminary study of the effect of ACTH preparations on the diabetogenic activity of various preparations of growth hormone (GH)

GH preparations derived from cattle pituitary glands

ACTH preparations (Armour & Co Batch nos 84-85H and 84-85U) derived from pig pituitary glands, administered in divided doses (3 injections/day) concurrently with the GH preparation but at a different site

in enhancing the diabetogenic activity of preparations of growth hormone, is itself a phenomenon with several puzzling features.

Effect of ACTH on the Diabetogenic Activity of Preparations of Growth Hormone

Fig. 7 summarizes the results obtained in a preliminary study of the effect of ACTH preparations on the diabetogenic activity of preparations containing growth hormone In these

to obtain a diabetic response in dogs with their own specimen of growth hormone. This material was dissolved at pH 10 in the present assays, because it was highly insoluble at neutral pH values. Raben and Westermeyer did not use alkaline solutions of their material, but injected either acidic solutions (pH 3·5) or mildly alkaline suspensions. In a preliminary experiment (Fig. 6) I gave their material at pH about 3·5,

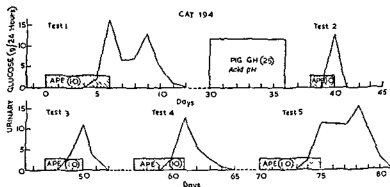


Fig. 6 Tests of a crude anterior-pituitary extract (APE), and of Raben-Westermeyer growth hormone (GH) given at an acidic pH, for diabetogenic activity

Period of treatment denoted [illegible]

and observed no glycosuria It would appear that diabetes cannot readily be induced in cats with growth hormone administered at an acidic pH.

Although no diabetes was induced by growth hormone (Raben-Westermeyer) thus administered in the experiment of Fig. 6, an unexpected result was obtained after the negative test—the cat was found to be hypersensitive to the diabetogenic action of the reference substance, namely an alkaline

to be more effective that "84–85II" with respect to this action in cats, has been found by Stack-Dunne and Young (1951) to be more effective in the so-called adrenal weight test, that is, in increasing the size of the reduced adrenal glands of the hypophysectomized rat. It is possible, then, that some factor quite distinct from the "ascorbic acid factor" is responsible for the potentiating action observed with ACTH preparations in cats. It is somewhat puzzling that the weight of the adrenals of hypophysectomized rats can be increased not only by ACTH preparations obtained from pig pituitary glands, but also, to an appreciable extent, by purified preparations of growth hormone from cattle pituitary glands (Stack-Dunne and Young, 1951). It would be premature to speculate whether there is any connection between this finding and the diabetogenic activity of growth hormone in cats.

I have obtained some support for the tentative conclusion I mentioned, that, with a given ACTH preparation, the diabetogenic potency of crude preparations of growth hormone is usually enhanced more than that of purified preparations. I performed successive experiments in each of three cats (Nos. 222, 229 and 231, Fig 8), with the same ACTH preparation throughout but with different preparations of growth hormone Serial experiments performed in other cats under these conditions, but with the same preparation of growth hormone throughout, indicated that the degree of potentiation was fairly constant in a given cat. It is thus particularly significant that, in each of three cats (Fig 8), ACTH enhanced the diabetogenic activity of a crude pituitary extract more than that of the purified preparations of growth hormone which were used in the preceding and subsequent experiments.

A possible explanation of this finding is that these crude extracts are relatively rich, as compared with the purified preparations, in some "co-factor" which enables, or at least helps, ACTH preparations to enhance the diabetogenic activity of growth hormone. A suggestive result has been obtained with a specimen of chorionic gonadotrophin (Fig 8, Cat 257) The diabetogenic potency of a purified preparation of growth

experiments the ACTH was given subcutaneously, concurrently with the growth hormone but at a different injection site. *We have been quite unable to demonstrate a diabetogenic action of ACTH alone, even with doses twenty times those employed in the present experiments, and even with a high-carbohydrate diet in place of our usual high-protein diet. Campbell, Davidson, Snair and Lei (1950) have obtained similar negative results in intact dogs, but it may be mentioned that a diabetogenic action of ACTH, given at a low dose, has been demonstrated in Houssay cats by Milman, deMoor and Lukens (1951).

We obtained very discouraging results in our first attempts to verify our initial hypothesis that ACTH could enhance the diabetogenic activity of growth hormone in the cat. In cats 229, 227 and 228, the ACTH preparation "84–85H" had little or no effect on the diabetogenic activity of a purified preparation of growth hormone (Fig. 7) I then repeated the experiments with the ACTH preparation "84–85U," and obtained definite indications of a potentiating action. The results were even more encouraging when I used crude preparations of growth hormone in place of a purified preparation. It appears, from the data of Fig. 7, that with a given ACTH preparation the diabetogenic activity of a crude extract of cattle pituitary glands is usually enhanced more than that of a purified preparation of growth hormone. Furthermore, it appears that, with a given preparation of growth hormone, the potentiating action of the ACTH preparation "84–85U" is greater than that of "84–85H," even if the latter is given at a higher dose in terms of potency in the conventional Sayers test.

I am not yet convinced that these differences between ACTH preparations *could not be due merely to differences* in the rate of absorption after injection into cats. At present, however, it appears that there is indeed a poor correlation between the potency of ACTH preparations in the Sayers test and their effectiveness in enhancing the diabetogenic activity of preparations of growth hormone. It is of interest in this connection that ACTH preparation "84–85U," which appeared

Resumé and Discussion

Preparations of growth hormone, derived from cattle pituitary glands, have been tested for diabetogenic activity in cats and for growth-promoting activity in rats. Even with preparations which had been subjected to drastic inactivating treatments, no indications have been found of a wide divergence between diabetogenic potency and growth-promoting potency. There was admittedly a tendency for diabetogenic potency to be rather higher than growth-promoting potency in the case of certain treated preparations. It appears, however, that the claim of Raben and Westermeyer (1952) that the diabetogenic factor is distinct from growth hormone is not based on valid evidence, and that the diabetogenic activity of preparations of growth hormone is due primarily to the same factor, or possibly group of factors, as is responsible for the growth-promoting activity of these preparations.

ACTH preparations derived from pig pituitary glands appear to be devoid of diabetogenic activity in the intact cat, but they can enhance the diabetogenic activity of purified preparations of growth hormone to some extent, and that of crude extracts of cattle pituitary glands to a considerable extent. It appears that the effectiveness of different ACTH preparations in producing this potentiation is not well correlated with their effectiveness in the conventional Sayers test. This potentiating action of ACTH in the cat may persist for some time after cessation of ACTH injections. Its mechanism has not been studied, but we have obtained no evidence for a lowering of the renal threshold for glucose in cats treated concurrently with ACTH and with preparations of growth hormone

These experiments were performed during the tenure of a Beit Memorial Fellowship I express my thanks to Professor F. G Young, F.R.S. [illegible]
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hormone was greatly enhanced if ACTH and the gonadotrophic preparation were administered concurrently, although not if only one of these was administered with the growth hor-

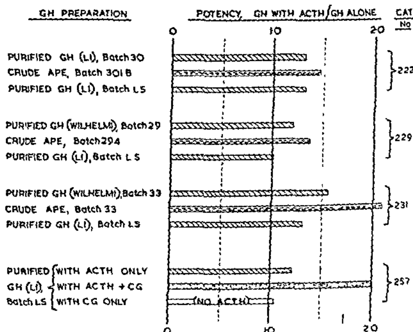


Fig 8 Further study of the effect of an ACTH preparation on the diabetogenic activity of various preparations of growth hormone (GH)

GH preparations derived from cattle pituitary glands; the

mone. It would, however, be premature to conclude that the pituitary co-factor, if indeed such a factor exists, is a gonadotrophin.

Resumé and Discussion

Preparations of growth hormone, derived from cattle pituitary glands, have been tested for diabetogenic activity in cats and for growth-promoting activity in rats Even with preparations which had been subjected to drastic inactivating treatments, no indications have been found of a wide divergence between diabetogenic potency and growth-promoting potency. There was admittedly a tendency for diabetogenic potency to be rather higher than growth-promoting potency in the case of certain treated preparations. It appears, however, that the claim of Raben and Westermeyer (1952) that the diabetogenic factor is distinct from growth hormone is not based on valid evidence, and that the diabetogenic activity of preparations of growth hormone is due primarily to the same factor, or possibly group of factors, as is responsible for the growth-promoting activity of these preparations.

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DISCUSSION

CONN In view of the recent reports of Raben and Westermeyer and

to us by Dr Raben He had found it to be growth-promoting but not

17-year-old male pituitary
Fig. 1 shows his photograph.
anhypopituitarism, namely,
cates the clinical data upon

L. P., Schoolboy, age 17.

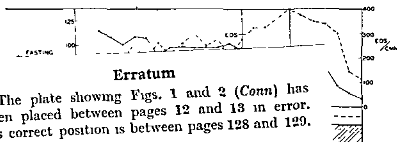
BMR—minus 23 per cent. Glycosuria ++++
G.T.T.-F—1050
Serum cholesterol—275 mg per cent 1h—344 ++++
h—301 ++++
h—205 ++++
l fields—Normal

Diagnosis: Calcified craniopharyngioma; panhypopituitarism with dwarfism; diabetes mellitus

FIG. 3 (*Conn*) Clinical findings in subject in Fig 1.

Raben's instructions, the material was found to be insoluble at neutrality but became completely soluble as the pH approached 4.

May I direct your attention first to the nitrogen balance data in Fig 4. In the base-line there was essential nitrogen equilibrium



Erratum

The plate showing Figs. 1 and 2 (*Conn*) has been placed between pages 12 and 13 in error. Its correct position is between pages 128 and 129.

Fig. 4 (*Conn*) Metabolic effects of Raben-Westermeyer growth hormone in man (L P, ♂, 17, panhypopituitarism, dwarfism and diabetes mellitus)

hormone

ame down Sharp nitrogen retention, however, returned Thus,

insulin administered to the point of maximal tolerability produced as much positive nitrogen balance as did the growth hormone. But that this appears to be a different kind of protein anabolism will be indicated in a subsequent Figure.

In Fig 5 we see 2 control glucose tolerance curves. Number 3 is that obtained after growth hormone There certainly was no intensification of the diabetic state

Fig. 6 is set up to show the nitrogen balance data in relation to those of phosphorus, potassium, sodium and chloride. It will be observed that during the period of growth hormone administration the positive

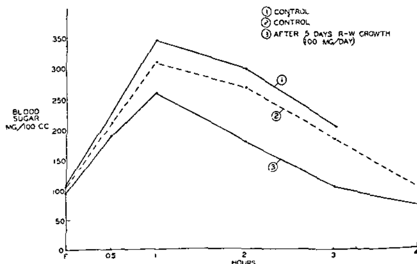


FIG. 5 (*Conn*) Effect of Raben-Westermeyer growth hormone upon glucose tolerance (L P., ♂, 17, panhypopituitarism, dwarfism, diabetes mellitus)

nitrogen balance was associated with concomitant retention of phos- [illegible] to be
[illegible] were
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[illegible] ature
[illegible]

[illegible]
hormone and that associated with excessive insulin activity. These data may help, however, to elucidate the recently reported findings of Dr Charles Best, our chairman, that administration of insulin induces

growth in hypophysectomized rats Our data are confirmatory with respect to total nitrogen retention but they suggest that the mechanism of protein synthesis under insulin may be different from that which is operative under the influence of growth hormone.

These observations may be summarized as follows:—

1. Significant anabolism of body protein has been observed in

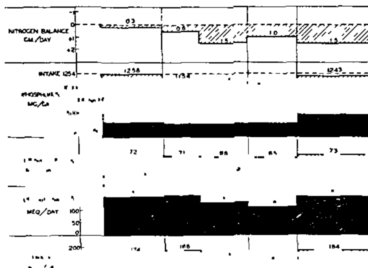


Fig 6 (*Conn*) Metabolic effects of Raben-Westermeyer growth hormone in man (L P , ♂, 17, panhypopituitarism, dwarfism, diabetes mellitus)

a growth-hormone-deficient-man given large doses of the Raben-Westermeyer growth preparation

2 No intensification of a pre-existing diabetic state was observed

3. Administration of insulin did not intensify the protein anabolic effect of the Raben-Westermeyer growth preparation

4. Insulin alone exerted a protein anabolic effect in this diabetic pituitary dwarf, who, without insulin, maintained nitrogen equilibrium But the associated data on inorganic metabolism suggest a form of nitrogen retention which differs from that produced by growth hormone.

5. From a practical point of view, it is believed that the Raben-Westermeyer preparatión, when given intramuscularly at pH 4, can be expected to induce growth in human pituitary dwarfism without the risk of inducing diabetes mellitus.

Before concluding these remarks I should like to show three figures from a study to which I had referred earlier. We had produced a "cleaned-up" crude anterior pituitary extract which when given intravenously to dogs produced severe diabetes mellitus. In retrospect this

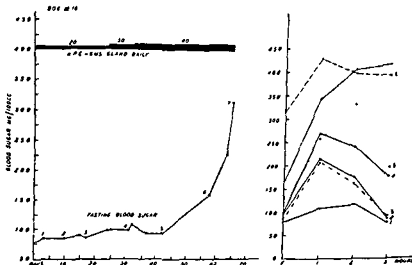


FIG. 7 (*Conn*) Fasting blood sugar curve of dog treated with "cleaned-up" crude anterior pituitary extract.

(shown at the right) were done. One notes decreasing tolerance for carbohydrate long before the fasting blood sugar begins to rise.

intensified the hypoglycæmia Fig. 9 shows the same phenomenon in the second patient.

These studies suggested that something in the extract stimulated increased release of insulin from the insulomas.

REID: I would add that in collaboration with Dr. Black and Dr. MacDougall, we have given purified preparations of growth hormone to a patient with hyperinsulinism, and have never observed hypoglycæmia, but we have observed a definite rise in the blood sugar.

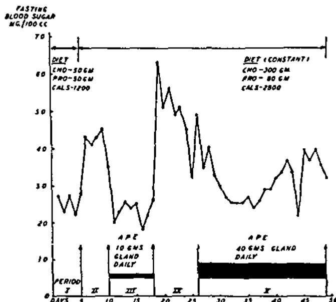


FIG. 8 (*Conn*) Fasting blood sugar curve in patient with a pancreatic insulinoma and hyperinsulinism, while being treated with same anterior pituitary extract as that given to dog in Fig. 7

Admittedly it wore off after a few days, but the effect did appear quite significantly

LAWRENCE I've met the same thing

MIRSKY Dr Conn, wasn't it a little dangerous to decide that that was a Houssay subject?

CONN Yes, one should not say Houssay, because the patient is neither completely hypophysectomized nor completely depancreatized.

MIRSKY: Well, that is exactly what I am thinking I've seen a patient similar to the last one, who had all the criteria of pan-hypopituitarism, as well as insulin resistance That patient required a large amount of insulin. Quite instantaneously, without any form of pituitary therapy, the insulin requirements dropped and the patient

began to grow If we had decided at that time that we were dealing with a Houssay preparation, we might have ended up with trouble.

CONN: I think there is no question in this individual that he has diabetes. The patient definitely has pan-hypopituitarism.

MIRSKY: I'd like to draw attention to an important aspect of this presentation—that a marked increase of nitrogen retention occurred when both insulin and growth hormone were administered; it was less with insulin alone

LUKENS I think both Dr. Reid and Dr. Conn would be interested

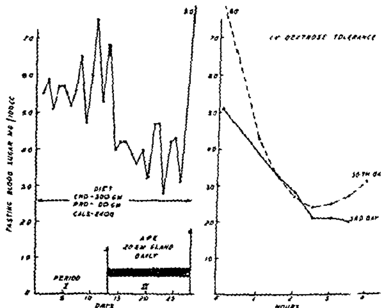


FIG 9 (*Conn*). Fasting blood sugar curve of a second patient with a pancreatic insulinoma and hyperinsulinism, while being treated with same anterior pituitary extract as that given to dog in Fig. 7

in hearing some preliminary remarks of Dr. de Bodo, who has been testing some of the Raben and Astwood preparations in hypophysectomized animals, for its effect upon insulin sensitivity. Now, if it is not diabetogenic, it ought not to counteract the insulin sensitivity of these animals It did counteract it, however, not quite as much as the conventional Li or Wilhelmi growth hormone, but to a significant degree. De Bodo's tentative thought was that this might be weaker as a diabetogenic agent.

CAMPBELL. I wonder if Dr. Conn has considered the possibility that the substance presented in his thesis was different from growth hormone?

CONN I have no idea whether it is different or not We were greatly impressed by the fact that this is one of the very few times that any

a pH of 10, and below a pH 4. In the range between pH 4 and pH 9, it is exceedingly insoluble. That raises the question whether, if the

to the continuing steady application of the growth hormone from the depot in the human, so that it may be that this difference in such a critical property may be related in a very important way to the responses observed.

CONN If that is true, there certainly seems to be a broad dose-response difference between nitrogen retention and diabetogenicity

CAMPBELL In that regard I think that Gaebler has shown that very small doses of the growth hormone have a pronounced nitrogen retention effect in dogs

PART III

THE INFLUENCE OF THE ADRENAL CORTEX ON CARBOHYDRATE METABOLISM

INFLUENCE OF THE ADRENAL CORTEX ON CARBOHYDRATE METABOLISM

C N H. LONG

THE participation of the 11-oxy adrenal cortical steroids in the regulation of carbohydrate and protein metabolism has now been well established. The experimental evidence in support of this has been gathered over the last twenty years, largely by observations made on the effects of adrenalectomy or of the injection of adrenal cortical steroids on the metabolism of fasting and fed animals, by observations on the effects of these procedures on phlorizin or pancreatic diabetes, as well as by studies on the temporary diabetes produced by the injection of large quantities of adrenal steroids or *ACTH* in intact animals.

Fasting Animals

Adrenalectomized animals, as well as individuals with Addison's disease, frequently develop hypoglycæmia when food is withheld. While the rapidity and severity of the hypoglycæmia is usually not as marked as that observed in hypophyseal deficiency, nevertheless the liver glycogen falls to very low levels and does not rise with continued fasting as it does in intact animals. The muscle glycogen is usually lower than that of normal animals but is not depressed to the extent found in animals deprived of the pituitary body.

In experiments where adrenalectomized animals are fasted and adequate renal function is maintained by administration of sodium salts, the urinary nitrogen excretion is some 20–30 per cent lower than that of normal animals, nor does it rise as the fasting continues

The injection of adrenal cortical extract or 11-oxy adrenal steroids into fasting adrenalectomized or hypophysectomized animals not only prevents hypoglycæmia but will raise the blood glucose above the normal level At the same time the liver glycogen stores are greatly increased but, in fasting animals, there is no accumulation of muscle glycogen. The resulting increase in the carbohydrate content of the body may be accounted for by the increased breakdown of tissue proteins since the urinary nitrogen excretion may be twice that usually found under these circumstances.

The same effects are also observed in fasting normal animals and indicate that the effect of these hormones is a direct one, and is not merely a consequence of the absolute or relative adrenal cortical insufficiency that is present in the former groups In all cases there is an accelerated loss of weight during the period of injection.

Fed Animals

Long, Katzin and Fry (1940) found that the injection of adrenal cortical extract or corticosterone into mice to whom a high carbohydrate diet was fed, resulted in a large deposition of liver glycogen (as high as 12 per cent) At the same time the muscle glycogen was increased. They also observed that the administration of cortical extract to glucose fed rats led to an increased accumulation of glycogen in liver and muscles, hyperglycæmia and mild glycosuria Several crystalline adrenal steroids were used instead of cortical extracts and of these 11-dehydro-17 hydroxy corticosterone was found to be the most active.

In chronic experiments, using rats force fed with a high carbohydrate diet, and injected daily with 11-dehydro-17-hydroxy corticosterone, Ingle (1941) found that marked

hyperglycæmia, and glycosuria occurred which disappeared on cessation of the injections.

In both the acute and long term experiments the nitrogen excretion was increased but under these circumstances the quantity of glucose in the urine could not be accounted for by an increase in tissue protein catabolism. This indicates that in addition to their capacity to stimulate gluconeogenesis these hormones, in the presence of adequate carbohydrate intake, also inhibit the utilization of this foodstuff by the tissues. Since there is no reason to suppose that the basic action of the hormones is any different in fed or fasted animals, it is probable that the greater degree of gluconeogenesis in fasting animals is merely a reflection of the smaller quantity of carbohydrate in the metabolic mixture furnishing the energy requirements of fasting animals.

Pancreatic Diabetes

Long and Lukens (1936) observed that adrenalectomy brought about alterations in the metabolism of total diabetes which in many respects were similar to those observed after hypophysectomy. In the fasting state, the chief alterations observed were a reduction in the blood glucose level with the not infrequent occurrence of hypoglycæmia, a marked reduction in glycosuria and ketonuria, and, in addition, a significant decline in urine nitrogen excretion Long, Katzin and Fry (1940) subsequently found similar effects of adrenalectomy on the diabetes of partially depancreatized rats, and demonstrated that the severity of the diabetes could be restored by administration of cortical extract and the 11-oxy adrenal steroids.

It would seem evident that at least part of the amelioration of pancreatic diabetes following hypophysectomy is a consequence of the reduction of adrenal cortical secretion that follows removal of the anterior lobe of the pituitary. Thus, Long and Lukens (cf. Long 1936–37) were able to show that the injection of pituitary extracts containing ACTH increased both the glycosuria and ketonuria of hypophysectomized-

depancreatized cats, while Lukens and Dohan (1938) reported that adrenal cortical extracts had the same effect. In view of the ability of both ACTH and the 11-oxy adrenal steroids to increase the rate of glucose formation from protein and to depress carbohydrate utilization, it is not surprising that the withdrawal of either of these hormones from diabetic animals would allow at least a partial return of carbohydrate metabolism to the normal state.

There are other conditions besides fasting and insulin deficiency that are associated with an increased rate of gluconeogenesis from protein. In some cases this is also accompanied by a reduction in the rate of carbohydrate utilization. Among these are phloridzin diabetes and an interesting effect produced by exposure of fasting animals to low oxygen tensions. The effect of adrenalectomy on phloridzin diabetes and on the response to low oxygen tensions has been reported by Evans (1936). In each case adrenalectomy was followed by a reduction in nitrogen excretion, and in phloridzinized animals by reduced glycosuria. In rats subsequently exposed to low oxygen pressures the usual accumulation of liver glycogen found in normal animals did not occur. Later, Wells and Kendall (1940) reported that the 11-oxy adrenal steroids restored the glycosuria and nitrogen excretion of adrenalectomized phloridzinized rats to the normal level.

Adrenal Cortical Hormones and the Cori Cycle

The experiments of Cori (1931) have shown that the subcutaneous injection of epinephrine into fasting rats is followed by a decrease in muscle glycogen, an increase in liver glycogen, a sustained hyperglycæmia and a rapid initial increase in blood lactic acid. The explanation given for these changes has been that epinephrine accelerates glycogenolysis both in liver and muscle and that the lactic acid released from the latter is the precursor of the liver glycogen and ultimately of the blood glucose. The reason for the accumulation of liver glycogen was believed to be due to the fact that glycogen synthesis was occurring in the liver at a greater rate than glycogenolysis.

However, it has recently been found that the injection of epinephrine also brings about discharge of ACTH from the pituitary (Vogt, 1944; and Long and Fry, 1945). Since the injection of adrenal cortical steroids also increases the liver glycogen of fasting animals the interesting question arises as to the participation of the anterior pituitary-adrenal-cortical system in the changes in carbohydrate metabolism that follow the injection of epinephrine.

Recently, my colleague, Dr. W. W. Winternitz, has compared the effect of epinephrine on the carbohydrate metabolism of normal and adrenalectomized animals. He finds that there are significant differences (Table I, A and B). The first point of interest is that after adrenalectomy the injection of epinephrine no longer brings about any significant increase in liver glycogen. This is in contrast to the nine- or ten-fold increase observed four hours after the subcutaneous injection of epinephrine in normal rats. Furthermore, the decrease in muscle glycogen is approximately twice as great as in adrenalectomized rats. These differences cannot be accounted for by any accumulation of glucose or lactate in the blood, since at the end of four hours the blood levels of both are substantially the same as in intact animals

In normal animals the carbohydrate loss from the muscles at the end of the four-hour period is almost balanced by the gain in liver glycogen and blood glucose. However, the effect of epinephrine injection in adrenalectomized animals is to leave at the end of the period a substantial portion of the carbohydrate loss from the muscle unaccounted for in these terms.

In further experiments, the effect of the injection of adrenal cortical extract before and during the period of action of epinephrine was studied. As Table I, C, shows, the injection of 1 ml. of extract every hour during the period of epinephrine action brought about a marked increase in liver glycogen over and above that produced in adrenalectomized rats by similar quantities of cortical extract. The increased loss of muscle glycogen was reduced. However, when the adrenalectomized

Table 1

EFFECT OF EPINEPHRINE ON CARBOHYDRATE METABOLISM OF FASTED NORMAL AND ADRENALECTOMIZED RATS

All values mg /100g /Body weight

	No rats	Liver glycogen		Muscle glycogen		Blood glucose		Net Change
		Amount	Change	Amount	Change	Amount	Change	
(A) Normal —Controls	6	5 ± 2	—	288 ± 10	—	30 ± 1	—	
,, +Epinephrine*	6	48 ± 5	+43	200 ± 19	-88	54 ± 3	+24	—21
(B) Adrex —Controls	6	2 ± 5	—	278 ± 6		22 ± 1	—	
,, +Epinephrine	8	8 ± 2	+6	112 ± 11	-166	50 ± 2	+28	—132
(C) Adrex —A C.E † (4 ml)	7	17 ± 1	—	256 ± 16	—	38 ± 2	—	
,, +A E C +Epinephrine	6	48 ± 5	+31	98 ± 8	—158	71 ± 3	+33	—94
(D) Adrex —A C E ‡ (8 ml)	5	42 ± 6	—	286 ± 16	—	38 ± 2	—	
,, + ,, Epinephrine	7	92 ± 4	+50	187 ± 9	—99	59 ± 3	+21	—28

**Epinephrine 0 02 mg /100 g B W /sub cut /4 hr period*

†Adrenal cortical ext 1 ml /hr for 4 hours

‡ ,, ,, ,, 1 ml every 2 hr for 12 hr before epinephrine injection and continued after

rats were pre-treated with cortical extract, and the injections continued after the injection of epinephrine, the liver glycogen increased by amounts similar to those observed in normal rats, while, in addition, the loss of muscle glycogen was reduced to the quantity found in normal rats (Table I, D). Consequently, the quantity of carbohydrate not accounted for at the end of the four-hour period was comparable to that found in normal rats injected with epinephrine alone.

While these experiments might be interpreted as another example of the inability of certain metabolic transformations to proceed in the absence of adrenal cortical hormones, they may also indicate the participation of these hormones in the reactions related to glycogen synthesis. It would appear from the experiments just cited that in the adrenalectomized animal the glycogenolytic action of epinephrine on muscle and liver glycogen proceeds to a greater degree than in normal animals. In the latter, epinephrine still augments glycogenolysis but the concomitant release of adrenal cortical hormones that it provokes may either inhibit this process or exert a retarding effect on some other phase of carbohydrate utilization such as the transformation of glucose to fat or the complete oxidation of glucose to CO_2 and H_2O. Furthermore, the influx of cortical hormones into the circulation may also add to the metabolic pool a quantity of carbohydrate derived from non-carbohydrate sources. Further experiments are necessary to determine which of these possible effects of cortical hormones is imposed on the primary action of epinephrine in normal animals.

The Mechanism of Action of Adrenal Cortical Steroids in Carbohydrate Metabolism

The alteration in the usual effect of epinephrine on carbohydrate metabolism that is observed in adrenalectomized animals, and the restoration of a normal response by the injection of cortical extract, raises questions of the interpretation that bear directly on the problem of the site of action of the adrenal cortical hormones.

Some years ago I prepared a simple diagram (Fig. 1) that will serve as a basis for the discussion of this matter. The experimental data available at that time did not allow more than the most general conclusions to be drawn and it is disappointing that even today not a great deal more can be added even though our knowledge of intermediary carbohydrate metabolism has been greatly advanced. Nevertheless, it may be worth while to indicate several possible sites of action if not for any other purpose than to see if any particular one best fits the experimental data

The various sites of action of the cortical hormones that

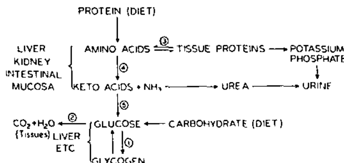


FIG 1 Possible sites of action of adrenal cortical hormones on protein and carbohydrate metabolism

have been suggested fall into two main groups (1) those that place their primary effect at some point in the metabolism of carbohydrate itself and which may be considered as a direct effect of cortical hormones on carbohydrate metabolism, (2) those that regard the primary effect of the cortical hormones as exerted on some phrase of protein metabolism. This is of such a kind as to bring about a considerable increase in the amount of carbohydrate precursors available for the processes of carbohydrate metabolism itself

(1) *Direct Effects on Carbohydrate Metabolism.* It is not easy from experiments on intact animals to state with certainty that the changes in carbohydrate metabolism following the

injection of such hormones as the adrenal cortical steroids, or epinephrine, are a consequence of the injected agent alone. It has just been pointed out that the alterations in the carbohydrate levels that follow the injection of epinephrine are rendered difficult to interpret by the release of ACTH. In addition, it is now known that increases in blood glucose of the magnitude that follow the injection of epinephrine into fasting animals are likely to be adequate stimulus for the secretion of additional insulin by the pancreas. It is also known that the three major pathways of glucose utilization, *that is, oxidation, polymerization to glycogen, and transformation* to fat are not only closely linked with each other but in addition are all catalysed by insulin

It is therefore not unlikely that the effect of cortical steroids upon these same three pathways of carbohydrate utilization may also be due to their relative inhibitory action on a metabolic process which is common to all. It is for this reason that the suggestion of Colowick, Cori and Slein (1947) that the adrenal steroids exert an effect on the hexokinase reaction is a particularly interesting one. These and additional studies have recently been reviewed by Krahl (1951), and they are so well known that it is unnecessary to quote them in any detail at this conference. It must however be pointed out that in experiments with isolated diaphragms the prior injection of the animals with cortisone or whole adrenal extract did not influence the glucose uptake. Indeed the only effect of such injections was to increase the depression of glucose uptake following treatment of the rats beforehand with anterior lobe extracts. It will be recalled, however, that the 11-oxy adrenal steroids will increase the liver glycogen of fasting, hypophysectomized rats; presumably therefore this effect on liver glycogen does not require the presence of any pituitary factor. Nevertheless, no accumulation of muscle glycogen was observed, and indeed most of the experimental evidence seems to implicate the liver rather than the muscles as the organ whose carbohydrate metabolism is most influenced by the adrenal cortical hormones.

Thus, Seckel (1940) and Corey and Britton (1940) reported that adrenal cortical extract inhibited glycogen breakdown in liver slices and in the perfused liver, while Buell, Anderson and Strauss (1936) as well as Koepf *et al* (1941) state that the liver of adrenalectomized rats cannot convert pyruvate and lactate to glycogen at a normal rate. In these experiments it may be pointed out that a failure to retain the glucose formed from pyruvate or lactate as glycogen could not be ruled out as the reason for the low glycogen levels observed If this were the case, then an inhibition of glycogenolysis could be the factor common to all the experiments quoted above

The explanation of all experiments on the effect of adrenal cortical hormones on carbohydrate metabolism must take cognizance of the fact that the urinary nitrogen excretion always rises as a result of their injection. If it be supposed that these hormones merely retard the release of liver glycogen as blood glucose then two secondary effects might be anticipated. First, the mean blood glucose level should decline, and secondly, the body would in consequence use a greater proportion of fat and protein for its energy needs The increased rate of protein catabolism would therefore be an indirect and secondary consequence of a primary effect of these hormones on glycogen metabolism Such an explanation could not account for the favourable effects of adrenalectomy on pancreatic diabetes, nor for the inability of adrenalectomized animals to supply sufficient carbohydrate for their needs under several circumstances that have been studied.

It is also important to reiterate that the administration of excess of cortical hormones to animals fed a high carbohydrate diet causes hyperglycæmia and glycosuria* and that the latter may be far in excess of the quantity that can be derived from the increased catabolism of protein This can lead to no other conclusion but that these hormones inhibit one or more of the usual metabolic pathways of glucose metabolism This may

*Although Conn, Louis, and Johnston (1949) suggested that ACTH lowered the renal threshold for glucose in man, Lambert and his colleagues (1951) were unable to observe any change

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the liver in the usual manner. This concept is also in keeping with the fact that replenishment of the depleted carbohydrate resources of adrenalectomized animals only retards but does not prevent the fatal consequences of the loss of these glands.

Nevertheless, the capacity of these hormones to make available the vast supplies of carbohydrate precursors locked in the proteins of the tissues, together with their additional *ability to retard carbohydrate utilization makes these agents* one of the most important factors in the protection of the nervous system against the consequence of hypoglycæmia.

When given in excess to intact animals, they not only present the organism with additional material to be converted *to carbohydrate but, at the same time, diminish its capacity* to dispose of this or dietary carbohydrate by the usual metabolic pathways. In consequence, the blood glucose may rise to levels at which glycosuria occurs.

In the adrenalectomized animal not only is there a diminished supply of carbohydrate precursors but the rate of transformation of carbohydrate into such substances as fatty acids appears to be accelerated Under these circumstances serious difficulties in maintaining an adequate blood glucose level soon appear and, along with them, a marked inability to resist any further circumstance or agent that tends to lower the blood glucose level.

The mode of action of these hormones, while far from complete in its details, emphasizes again the close interrelationships between the metabolism of protein and carbohydrate and furnishes an excellent example of the integration of the endocrine system with the processes of metabolism; an integration which is directed towards the preservation of the most optimal conditions for cellular function

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be, as the experiments of Welt and Wilhelmi (1950) suggest, the transformation of glucose to fatty acids or it may be at some other point in the intermediary metabolism.

(2) *Direct Effects on Protein Metabolism.* It might be anticipated that if there are still difficulties in locating the point of action of adrenal cortical hormones on carbohydrate metabolism, then attempts to define the nature of their effect on protein metabolism would encounter even greater obstacles. We possess a considerable body of knowledge concerning the intermediary transformations of glucose which is not matched to any degree by our understanding of the processes of tissue protein synthesis and degradation Nevertheless, an impressive case can be made for the direct intervention of the adrenal cortical hormones in tissue protein metabolism.

In the first place the continued injection of adrenal cortical hormones into normal fed animals causes loss of body weight and increased nitrogen excretion without the occurrence of glycosuria Furthermore, several growth processes such as wound repair, the response of connective tissue and the growth of cartilage and bone are retarded by these hormones. Their effects on lymphoid tissue which are both rapid and highly destructive and are paralleled by their capacity to reduce the number of circulating eosinophils Other examples can be cited, but the general impression is that these hormones exert a catabolic (or anti-anabolic) effect on the protein metabolism of a large number of tissues. Whether the effect on protein metabolism is a primary one or merely secondary to some other damaging influence of these substances on cellular function cannot be decided at the present time.

In a certain sense this accumulation of liver glycogen may be regarded as a secondary consequence of the effects of cortical hormones on metabolism. Bondy (1949), Kline (1949), Hoberman (1950) and Engel (1951) have shown, by various techniques, that the rate of tissue protein breakdown and amino acid release is accelerated by cortical hormones and occurs in the absence of the liver. This furnishes a plethora of amino acids which in the intact animal are disposed of by

had been any marked effect on the oxidation, one would anticipate that the respiratory quotient would rise as it does in fasting hypophysectomized animals

Drury. The R.Q. would depend on what the ultimate fuel is.

Long In fasted hypophysectomized animals, especially the rat, you get a fasting R.Q of about the order of 0 85, as against 0 72 which you get in the normal animal An adrenalectomized animal, in our experience, does not show that high R.Q during fasting. There is certainly a difference there between the adrenalectomized and hypophysectomized animal.

have the fact in the fasting animal that you are presenting the liver with more precursors It has always been a paradoxical effect to my mind that a fasting animal can accumulate 10 or 20 times as much glycogen as normally in a 12-hour period when one presumes that its need for carbohydrate would be greater It doesn't appear to be able to utilize this accumulation in a normal manner

Conn I have a comment with respect to the epinephrine situation. Some years ago, we studied in normal men, by continuous indirect calorimetry in an open-circuit system, the effect of epinephrine on the total non-protein respiratory quotient We found that on a fixed preparatory diet, 22 per cent of the total calories were derived from carbohydrate in a four-hour period in the fasting individual On another day under the same conditions but with a test dose of glucose, the percentage of calories from carbohydrates rose to about 50 per cent.

was given epinephrine his total R Q dropped below his fasting level That is suggestive, as Dr Long has pointed out, that oxidation of carbohydrate, or conversion of carbohydrates to fat, or both, is inhibited by the epinephrine, or by the adrenal cortical hormone.

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DISCUSSION

[illegible] of the effect of ACE on

[illegible]

animals, in eviscerated adrenalectomized animals and in eviscerated animals given cortisone, and have found no difference in any of them in the production of CO_2 from glucose We've come to the conclusion, therefore, that ACE has no effect on the extra-hepatic tissues as regards the oxidation of glucose, and the effect would have to be looked [illegible]

[illegible]

an accumulation of carbohydrate in the blood, I do not think we know. I'm very interested to hear that your studies in the peripheral tissues [illegible]

largely depends on the circumstances—whether the animal was fasting or not, the amount of carbohydrate that was fed, or whether it was the post-absorptive or the absorptive period.

G. CORI Or the period after injection. The time factor is very important

SHERLOCK We've performed hepatic biopsy before starting the epinephrine and then while infusing the epinephrine for one hour After half an hour of infusion the liver glycogen was diminished. It was still lowered after one hour It returned to pre-infusion levels one hour after the epinephrine was stopped, but did not increase above the initial values (Hildes, J. A , Sherlock, S , and Walshe, U , 1949, *Clin Sci* , 7, 297)

[illegible]

sensitive.

on gluconeogenesis, with increase in carbohydrate formation, and the effects on utilization of carbohydrates. The latter may be controlled by growth hormone, either alone or acting with the adrenal cortical hormone As far as I know, little has been done with the cortical steroids in hypophysectomized animals to show whether there is an effect of cortical steroids on carbohydrate utilization in the absence of growth hormone. I know of no critical experiments on that score. When cortical steroids have been given in a few instances, the effects have all been explicable on the basis of a gluconeogenic effect. Do you know of any such experiments?

Long: No, I don't, beyond the experiments I have shown. Of course, starting with the whole adrenal extract, presumably the effects were due to 11-ox
liver glycoge
presumably
way in these animals.

G. Cori. Walaas and Walaas have shown that epinephrine does
factors,
cluded
tuitary
hormone, also does, by itself, inhibit the uptake of sugar by the muscle, though weakly

Turner In New Zealand they have a problem of temporary underfeeding in dairy cattle, associated with a reduction in the protein content of milk It occurred to me that it might be due to an increase in the adrenal hormone output causing increased de-aminization and less protein precursor available. Do you think that that might be a likely explanation of the phenomenon?

Long . It's hard to say.

Turner I should have mentioned that you indicate an increase in the blood-amino acids, but does that exist under intense lactation? I wonder, Dr Folley, if there might not actually be a deficiency of circulating amino-acids and the synthesis of milk low in protein?

Folley I wouldn't like to give an opinion on that here and now. I think that is a problem which needs some thinking about

Sherlock: How quickly does adrenal cortical extract increase liver glycogen?

Long Perhaps the best answer to that, Dr. Sherlock, is that, as you know, this deposition of liver glycogen has been used in assay methods in this connection. From our limited experience, in the rat at any rate, it is certainly an hour before you begin to get any increase, and it is not well-marked until 4 hours after injection

... before and after

injection?

Long: No—I'm afraid I have no evidence as to what the level of liver glycogen is going to be after epinephrine injections I think that

The idea that adrenal cortical activity is connected with *phosphorylating enzymes (Verzár, 1939)*, resulted from the observation that selective carbohydrate absorption decreases in the intestine after adrenalectomy. We shall not discuss this here now, but might mention that it has been confirmed again recently.

The first proof that alkaline phosphatase decreases rapidly after adrenalectomy was shown by chemical methods by Kutscher and Wust (1942) on guinea pigs. In ten animals after adrenalectomy the alkaline phosphatase decreased to about half, and in seven animals with DCA it became normal again Then Verne and Hébert (1948, 1949) showed with the histological method of Gomori (1939) that the alkaline phosphatase disappears in the epithelial cells of the intestinal *mucosa. This was confirmed by Soulairac (1948), Stenram* (1951) and ourselves (Verzár, Sailer and Richterich, 1952). Using glycerophosphoric acid at *p*H 9·2 on homogenates of the mucosa of the upper 20 cm. of the small intestine of rats we found 31·2 mg. P/h in normal rats, and 19·6 mg. P/h in adrenalectomized ones ($p = < 0·01$), 89 normal and 59 adrenalectomized animals were used. The phosphatase decreased if adynamia appeared. We emphasized especially that a decrease of alkaline phosphatase was also seen in animals kept for a long time in a healthy state with 1 per cent sodium chloride (Fig 1). We saw a decrease from 100 per cent to 55·2 per cent in 21 normal animals and 31 adrenalectomized animals between the 26th and 63rd day after adrenalectomy.

Daily treatment with 1 or 2 mg. DCA or 1 mg. cortisone, or 0·5 ml. Eschatin twice daily kept the phosphatase activity normal DCA was already active after 12 hours, and cortisone if given three times in 24 hours acted similarly. It seems that the action of DCA is longer lasting than that of cortisone, which appears and disappears quickly

A large number of these animals were also studied with the histological method of Gomori Several authors have shown the distribution of phosphatase in the mucosa itself. The *highest concentration is in the striated border (Figs. 2 and 3).*

ADRENAL CORTEX AND CARBOHYDRATE PHOSPHORYLATION

F VERZÁR

In this review I wish to concentrate on the actual studies of phosphorylating enzymes in relation to the adrenal cortex. I do not intend to discuss carbohydrate metabolism in this connection at all, because this will be done at the Symposium on "Experimental Diabetes" in Leiden, 1952. I shall limit my discussion to phosphorylating enzymes of the intestinal mucosa, the kidney, the liver and the muscle.

I

The *intestinal mucosa* contains the highest concentration of alkaline phosphatase in the body; here, according to Greenstein (1945) it corresponds to 2,789 units/g, in the kidney to 1,072, in the liver to 4 and in skeletal muscle to 2 units/g.

There is, I think, today no doubt that selective carbohydrate absorption is the result of a hexokinase-phosphatase activity. Originally this idea was conceived through the observation that poisons like phloridzin inhibit selective glucose and galactose absorption. Bruckner (1951) has lately compared the absorption of mixtures of galactose and sorbose, and shown that phloridzin in low concentration inhibits only absorption of the former. Atebrin and dinitrophenol, however, do not, and it might be discussed whether one has to conclude that the phosphorylating part of the reaction is the important part. Hele (1950) a short time ago measured the hexokinase activity parallel to the absorption of different sugars A main proof that sugars are phosphorylated in the intestinal mucosa is that they are found as phosphoric acid esters in the mucosa *during absorption (Laszt and Sullmann,* 1935; Beck, 1942; Kjerulf-Jensen, 1942).

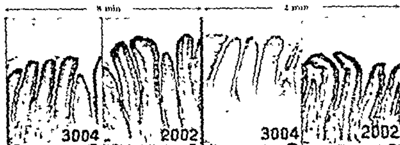


Fig. 2 Low magnification

Fig. 3 High magnification

On the left the preparations were incubated for eight minutes, on the right for two minutes with glycerophosphate

3004 and 3003 in Fig. 2 and 4004 and 4002 in Fig. 3 are from adrenalectomized animals

2002 and 2000 in Fig. 2 and 1000 and 1004 in Fig. 3 are from normal controls

To face page 154]

Next follows the Golgi apparatus, but there are certainly black granules in these preparations in the cell protoplasma between the striated border and the nucleus. The nucleus shows some phosphatase only after longer incubation. There are observations which point to the possibility that these are different phosphatases.

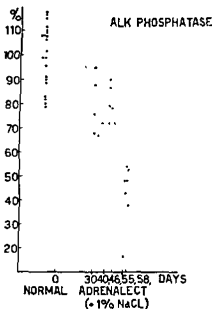


FIG. 1 Alkaline phosphatase content of the mucosa of the small intestine in normal animals and in adrenalectomized animals, both groups being given 1 per cent NaCl

We have especially emphasized the necessity to incubate the slices for a short time only, in order to get reliable results, because of the great activity of this phosphatase. We have completely confirmed the findings of Verne but we have to emphasize that the striated border might still contain large amounts of alkaline phosphate while the cell body has already lost it. Restoration with adrenal corticoids was also shown.

II

Kidney has the second highest activity of alkaline phosphatase in the body. This is localized in the reabsorbing epithelial cells of the convoluted tubules. These cells have a similar histological structure to those of the intestinal mucosa, especially in their cuticular border. They transport glucose actively into the blood by phosphorylation and are inhibited with phloridzin (Lundsgaard, 1933, Kalckar, 1938).

It was supposed by the author (Verzár, 1939) on the basis of clinical observations (Thaddea, 1936) that this epithelium loses its alkaline phosphatase activity after adrenalectomy. Jimenez-Diaz (1936) had already found a decrease of alkaline phosphatase in the kidney of the cat after adrenalectomy. Kutscher and Wust found this decrease in 1942 in adrenalectomized guinea pigs and its restitution with DCA, and Vail and Kochakian (1947) confirmed it but claimed to prevent it by including 1 per cent NaCl in the drinking water. Kochakian found that testosterone, which produces kidney hypertrophy, increased the alkaline phosphatase also (as well as acid phosphatase). Folley and Greenbaum (1946) found the same in rats and that restitution was possible with adrenocortical extracts and with DCA (3 mg./day) and also cortisone. Tissières (1948) also found it—he estimated phosphomonoesterase activity with glycerophosphate at *p*H 9; the decrease was from 41·1 to 24·8 per cent ($p < 0 \cdot 01$); for pyrophosphatase activity at *p*H 8 0 he found a decrease from 11·4 to 6·4 per cent ($p < 0 \cdot 01$). No changes were observed with either of these enzymes at a lower *p*H. (His results on the intestine were negative since he used whole intestine instead of intestinal mucosa and overlooked therefore the great changes in mucosal activity.)

Furthermore, Tissières used Gomori's histological method on the kidney. The alkaline phosphatase of the convoluted tubules disappeared after adrenalectomy. Curiously enough the glomeruli, which are normally quite untainted, become dark in these cases. This mi [illegible] the result of [illegible]dal

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phosphatase liberated in the tissues after adrenalectomy and kept back by the glomerular membrane, but this has not been studied further. Kochakian (1948) saw similar pictures after large doses of different steroids.

Vail and Kochakian (1947) found no influence on the acid phosphatase of the kidney.

III

Phosphorylase activity in the *liver* has been studied by two teams in our laboratory. Doetsch (1945) used the method of Ostern, Herbert and Holmes (1939): 1 ml. 2 per cent glycogen solution, 1 ml. 0·01N NaF, 1 ml phosphate buffer (Sorensen), pH 7 17, and 0·5 g. liver mush were mixed and deproteinized after 30 minutes with trichloracetic acid. The same method was used by Staehelin and Vogtli (1947). These authors increased the fluoride concentration to 0·04N. The following table shows their results:—

Table I

PHOSPHORYLASE ACTIVITY OF LIVER

Author	*Rats*	*No of Animals*	*mg P per 100 ml decrease after*		
			7	15'	30'
Doetsch (1945)	normal	18	—	26 2	33 8
	adrenalect.	16	—	7 8	15 0
Staehelin and Vogtli (1947)	normal	39	10	22	35
	adrenalect.	24	8	12	24

The alkaline phosphatases of liver have a very low activity. Kochakian and Vail (Kochakian and Vail, 1944; Vail and Kochakian, 1947) also saw a decrease after adrenalectomy and an increase again after treatment with 11-oxycorticoids. In the mouse, however (Kochakian and Robertson, 1950, p. 492), found it only much delayed 7 to 30 days after the beginning of treatment with cortisone pellets; they related their findings to those of Fraenkel-Conrat, Simpson and Evans (1943), and of Folley and Greenbaum (1946), who had described a decrease

in arginase activity in the liver after adrenalectomy in rats and an increase with corticoids. Kochakian and Robertson's opinion is: "If . . . an intense acute gluconeogenesis is stimulated by these steroids, no concomitant increase in arginase activity of the liver of either adrenalectomized or normal rats occurs, but the alkaline phosphatase is greatly increased." Neither the increasing arginase level nor the level of alkaline phosphatase runs parallel with the rapid decrease of body weight and glycogenesis observed.

IV

In minced *skeletal muscle* and *heart muscle* of rats, Schumann (1940) was the first to show that after adrenalectomy phosphorylase activity is decreased. Phosphorylase adds inorganic phosphate to glycogen, producing glucose-1-phosphate as the first product of glycogenolysis. Lohmann's method was used. To minced muscle, 1 per cent of glycogen and NaF was added and the decrease of inorganic phosphate followed. The experiments were done at *p*H 8 2 and 20°C Montigel and Verzár (1943–45), Doetsch (1945), and Stachelin and Vogtli (1947) repeated these experiments and studied the influence of different steroid hormones. It was found, as shown in Table II, that phosphorylase activity is decreased after adrenalectomy

A similar decrease of glycogen phosphorylation was found in skeletal muscles of 8 adrenalectomized cats and 2 adrenalectomized dogs (Montigel and Verzár, 1943) With Stachelin and Vogtli (1948), adrenalectomized cats were kept alive by daily treatment with 5 mg. DCA, then a piece of the rectus femoral muscle was excised and tested for phosphorylase activity. After the wound healed, the animal was brought into a crisis by discontinuing DCA treatment, and phosphorylase activity was again estimated in the parallel muscle. It decreased significantly.

DCA restored the decreased phosphorylation activity *in vitro* in quantities of 0 02 to 2 mg./100 g. muscle. Progesterone acted similarly but in 20 times higher concentrations Also

cysteine and glutathione increased the phosphorylase activity of *adrenalectomized rats' muscle which is of special* interest since Williams and Watson (1940) found a similar increase for kidney and bone phosphatase, and G. Cori in 1945 also with purified phosphorylase.

The *in vitro* restoration of phosphorylase activity was not

Table II

PHOSPHORYLASE ACTIVITY OF MUSCLE

Author	*Rats*	*No of Animals*	*mg P per 100 ml decrease after*			*T* –
			7′	15′	30′	
Montigel and Verzár (1945)	normal	10	—	52 6	67 6	—
	adrenalect	45	—	33 5	50 1	—
Doetsch (1945)	normal	32	—	41 6	59 4	4 1 (15′)
	adrenalect	57	—	25 4	44 1	4 5 (30′)
Staehelin and Vogtli (1947)	normal	20	24	45	65	7 0 (15′)
	adrenalect	63	16	31	52	6 6 (30′)

successful in some of the later experiments and obviously we *do not know all conditions of this reaction.*

Cori (1946) studied hexokinase activity with pure enzymes. Its activity was inhibited by ACTH and adrenal cortical extract. Smith and Young (1949), Stadie and Haugaard (1949) and Broh-Kahn and Mirsky (1947) were unable to confirm this.

Phosphoglucomutase was studied by Conway and Hingerty (1946) They gave a complete analysis of the carbohydrate metabolites of skeletal muscle of normal and adrenalectomized rats. These differed mainly in the concentration of the hexose esters. The following is an extract of their table:—

	Normal m mol kg	Adrenalectomized m mol kg	Change Per cent
Total hexose monophosphate-P	10.04 ± 0.87	5.67 ± 0.46	−43.5
Glucose-1-phosphate-P	2.74 ± 0.21	3.68 ± 0.19	+34.3
Glucose-6-phosphate-P	6.42 ± 0.83	2.06 ± 0.16	−67.9
Fructose-6-phosphate-P	0.94 ± 0.06	0.34 ± 0.03	−63.8

There was no change in total P, acid soluble P, ATP, fructose-1,6-phosphate-P, triosephosphate P, phosphopyruvic acid P, carnosine, anserine and phosphocreatine P.

The conclusion was drawn that the glucomutase activity decreases after adrenalectomy. This was supported by Keyes and Kelley (1949) on the basis that adrenal cortical extracts increase plasma lactate and pyruvate, together with the glycogen content of the liver

Acid phosphatase of muscle does not decrease after adrenalectomy of guinea pigs (Knoevenagel, 1940).

The increase of phosphorylase activity through corticoids means an increase of glycogen breakdown in the muscle. Also Cori's finding of an inhibition of a hexokinase by corticoids would have the same meaning This seems to be in agreement with several other observations of an antagonism of the corticoids with insulin. So far nobody has been able to show an increase of glycogenetic enzyme activity in muscle.

V

It is questionable whether the rôle of the phosphorylating enzymes in connection with corticosteroids is a specific one It is certain that other enzymes are also influenced but their number is limited. Two groups can be differentiated which change after adrenalectomy and are influenced by corticoids. (*i*) *Protein metabolic enzymes.* *Jimenez-Diaz (1936), Russell* and Wilhelmi (1941) found that after adrenalectomy amino acid d-aminase decreases in the kidney but not in the liver, G. F. Koepf in 1941 and Leupin (1950) with muscle found no alterations. (*ii*) Arginase activity of the liver: this decreases

after adrenalectomy and is restored by 11-oxycorticoids (Fraenkel-Conrat, Simpson and Evans, 1943). Folley and Greenbaum (1946, 1948) showed the same decrease in liver, kidney and mammary gland; they restored the activity with deoxycorticosterone as well as with 11-oxycorticoids and proved that anorexia was not the cause of this decrease. Kochakian found that arginase decreased, and in his paper with Robertson (Kochakian and Robertson, 1950) he showed that arginase activity can be restored with ACE and with cortisone in the liver as well as in the kidney. He also found a decrease of *alanine-D-aminase and glutamine-D-aminase* in the kidney and not in the liver of rats

The other group of enzymes connected with the corticoids are oxydases According to Tipton (1940), liver slices of adrenalectomized animals oxidize pyruvate and lactate less than those of normal animals. Gordan and Elliott (1947), Eisenberg *et al* (1950), Hayano, Schiller and Dorfman (1950), described that the oxygen uptake of brain tissue is inhibited by DCA. Gordan *et al*. (1951), p. 576, show maximal inhibition with deoxycorticosterone and stilbœstrol and less with other corticosteroids. This is very similar to the order of activity of inhibition of glycogenesis in muscle which we had found with Wenner (Verzár and Wenner, 1948). Gordan believes that the steroids interfere with the pyruvate in the Krebs cycle before and after dehydrogenase activity related to flavoprotein They mention in this connection that Kun and McCurley (1950) found that steroid hormones activate the formation of phosphoenoly-pyruvate from 3-phosphoglycerate

Directly related to these findings are those of Hayano, Dorfman and Yamada (1950) who found that DCA inhibits tyrosinase and generally D-amino-oxidases. Other steroids were much less or not at all inhibitive. D-Aminoacidoxydase with D-alanine as substrate is a flavoprotein. The co-enzyme is alloxazine-adenine-dinucleotide. Acetone powder of pig kidney was used and they have lately (Hayano and Dorfman, 1951) explained· "An increase in the aqueous solubility of

DCA by virtue of a combination with the apo-enzyme at a point necessary for enzyme activity appears to occur. Since the inhibition can be relieved by acetone precipitation with a recovery of essentially all the original activity, no change in the apo-enzyme occurred."

A third step in this line is the work of Umbreit (Umbreit, 1951; Umbreit and Tonhazy, 1951), who actually showed that adrenalectomy decreases oxidation of glutamine and proline by kidney homogenates, and cortisone increases, i.e restores this, he then found· "The other alteration in adrenalectomized animals is an increased requirement of adenilic acid in order to maintain the maximum rate of oxidation. This increased requirement for adenylate is the cause of the decreased oxidation in homogenates from adrenalectomized animals of substances such as α-ketoglutarate" We do not understand, however, why cortisone increases proline oxidation in kidney and not in liver but cortisone increases D-aminoacid oxidation in liver and not in kidney.

Phosphorylating enzymes do not act in isolation. Their function is intimately connected with the oxidation cycle. If protein is the source of carbohydrate, they are also connected with D-aminase activity.

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DISCUSSION

LONG. What do you mean by the adynamic adrenalectomized animal? Do you mean adrenal insufficiency?

VERZÁR. Yes. We use the following test The animals climb on a steep wire net, if they let themselves fall back, we call them "adynamic." Six days after adrenalectomy, without treatment, rats often

spite of the long survival, glucose-absorption had decreased. That just proves that the criticism, that these adrenalectomized animals might not have been in the best state, is not relevant

BEST: It is a little difficult to think of perfect control, isn't it?

RUSSELL: One other thing that might be controlled is the food intake of the animals Adrenalectomized animals on salt, in my experience, don't like some things and unless you pay special attention to the composition of the diet, they have often a rather low food intake. It has been shown in normal animals that with reduction in food intake there is reduction in the rates of absorption of carbohydrates. I was wondering if measurements have been made of food intake in the adrenalectomized animals, and whether the controls have been given the same quantity, or was it shown that these salt treated animals did have a normal food intake?

VERZÁR Marazzi's experiments were done on a rather limited number of animals and are not convincing NaCl-kept adrenalectomized rats keep up their body-weight or even increase it.

RUSSELL I've had the same experience.

VERZÁR In a large series of NaCl-kept, adrenalectomized rats we

and they still will survive Obviously they have regenerated interrenal tissue

FOLLEY Could I ask Professor Verzar if he can speculate at all on

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DISCUSSION

Long: What do you mean by the adynamic adrenalectomized animal? Do you mean adrenal insufficiency?

Verzár: Yes. We use the following test: The animals climb on a steep wire net, if they let themselves fall back, we call them "adynamic." [illegible]

Verzár: These are NaCl-treated, adrenalectomized animals In spite of the long survival, glucose-absorption had decreased That just proves that the criticism, that these adrenalectomized animals might not have been in the best state, is not relevant

Best: It is a little difficult to think of perfect control, isn't it?

Russell: One other thing that might be controlled is the food intake of the animals. Adrenalectomized animals on salt, in my experience, don't like some things and unless you pay special attention to the composition of the diet, they have often a rather low food intake. It has been shown in normal animals that with reduction in food intake there is reduction in the rates of absorption of carbohydrates. I was wondering if measurements have been made of food intake in the adrenalectomized animals, and whether the controls have been given the same quantity, or was it shown that these salt treated animals did have a normal food intake?

Verzár: Marazzi's experiments were done on a rather limited number of animals and are not convincing NaCl-kept adrenalectomized rats keep up their body-weight or even increase it

Russell: I've had the same experience.

Verzár: In a large series of NaCl-kept, adrenalectomized rats we measured [illegible] tissue.

Folley: Could I ask Professor Verzár if he can speculate at all on the rôle of the alkaline phosphatase in the intestinal mucosa?

Young: I think the answer to that question is that it catalyses the hydrolysis of the glucose-6-phosphate that may be formed under the influence of hexokinase during the absorption of glucose from the gut. I hope Dr. Dorothy Needham will agree with me.

NEEDHAM: Yes, I think Miss Hele in her experiments, although she found the hexokinase activity with the phosphorylation of fructose and glucose corresponded to their rate of absorption, did not find any difference in the rate of de-phosphorylation of the 2 esters.

VERZÁR: Hexokinase-activity after adrenalectomy has not been studied in the intestinal mucosa, but phosphate esters of glucose and galactose have been shown to appear during absorption of these sugars in the mucosal cells. The alkaline phosphatase could have the second activity of splitting these esters, since in the blood plasma we find the free sugars again.

YOUNG: On this question of food intake, did you know that the adrenalectomized animals were in fact absorbing the same amount of food as the controls?

VERZÁR: Since they were growing or kept their weight, we could suppose this.

LONG: I would just like to ask one more question which Professor Verzár might think a little provocative. Do you regard deoxycorticosterone and cortisone as equivalent in their effect on carbohydrate metabolism, except for the question of time involved?

VERZÁR: In my articles in the American Journal of Physiology, 159, 256, 263 (1949), I stated that their carbohydrate activity is not equivalent. The whole question is at present in a new state. DCA is promptly oxidized to 11-oxycorticoids in the adrenals (Pincus) and also in the liver, kidney and other organs (Kahnt and Wettstein, 1951). Why should we suppose then that DCA which we inject in an adrenalectomized animal, is not also transformed to 11-oxycorticoids?

LONG: But then he hasn't got an adrenal gland to do it.

VERZÁR: This is not necessary, since liver and kidney oxidize DCA also.

LONG: What puzzles me about this is that in some of the experiments you showed, in order to get an effect you apparently had to give a total of 5 mg. a day of DCA in divided doses.

VERZÁR: No. The slide showed an experiment in which DCA was given in small quantities every 6 hours (Wirz, 1950, 1951).

LONG: [illegible]
in my exp [illegible]
the amou [illegible]
of the fac [illegible]
deoxycorticosterone does not protect the animal, for example, against insulin. In fact, according to Sayers, animals are even more sensitive to the hypoglycæmia of insulin when they are treated with deoxycorticosterone, than they are without it. I think that Sayers showed that regardless of the amount of deoxycorticosterone which he gave, he could not get protection of the animal against circumstances [illegible]

protective action which one can get with adrenal cortical extract or with cortisone.

VERZÁR Peyser in 1941 showed an anti-insulin effect of DCA on mice. I think that there can be no doubt that DCA is not one of the physiologically-produced hormones and probably cortisone isn't either.

ALLAN I should like to mention that in people with Addison's disease, DCA does not stabilize the blood sugar (i.e. fails to protect against hypoglycæmia)

LAWRENCE I can certainly add clinical evidence to that. In a severe diabetic who developed Addison's disease, his mineral metabolism was rectified by DCA and he became intensely sensitive to insulin Instead of requiring 60 units a day, we could only give him 2 units in the morning and 3 in the evening, and even that would produce hypoglycæmia

CONN I think that the clinical observations mentioned are well-established, but there is another observation which I think one should mention in this connection. I agree with Dr Long that there is very

metabolic effects

VERZÁR I might mention that just a short time ago we tried to keep adrenalectomized rats at low oxygen pressure (350 mm Hg). DCA did not protect the animals But if we gave daily 1 5 mg DCA with 0 5 mg cortisone, we could keep them continuously alive with normal body temperature at this low pressure This agrees with the clinical observations

NEEDHAM Yes, I think Miss Hele in her experiments, although she found the hexokinase activity with the phosphorylation of fructose and glucose corresponded to their rate of absorption, did not find any difference in the rate of de-phosphorylation of the 2 esters.

VERZÁR Hexokinase-activity after *adrenalectomy* has not been studied in the intestinal mucosa, but phosphate esters of glucose and galactose have been shown to appear during absorption of these sugars in the mucosal cells. The alkaline phosphatase could have the second activity of splitting these esters, since in the blood plasma we find the free sugars again.

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VERZÁR No The slide showed an experiment in which DCA was given in small quantities every 6 hours (Wirz, 1950, 1951)

LONG: Well, the point I am trying to make, Professor Verzár, is that in my experience and I think in the experience of others, regardless of the amount of deoxycorticosterone which you give, and regardless of the fact that there may be transformation, the administration of deoxycorticosterone does not protect the animal, for example, against insulin. In fact, according to Sayers, animals are even more sensitive to the hypoglycæmia of insulin when they are treated with deoxycorticosterone, than they are without it I think that Sayers showed that regardless of the amount of deoxycorticosterone which he gave, he could not get protection of the animal against *circumstances* [illegible]

protective action which one can get with adrenal cortical extract or with cortisone.

insulin activity may be due to diminished pancreatic production, increased insulin destruction, or depression of insulin activity by antagonistic substances. Such considerations lead inevitably to the conclusion that the inciting factors which initiate the diabetic state in man may be multiple and variable. With these thoughts in the background one may attempt to evaluate the influence of adrenal cortical steroids upon carbohydrate metabolism in man

For many years it has been known that about 70 per cent of patients with Cushing's syndrome exhibit a type of diabetes mellitus which is relatively insulin resistant. It is now recognized that Cushing's syndrome represents the multiple manifestations produced in man by a continuous over-production of adrenal cortical steroids of the 11-17 oxygenated type. In many of such patients total or sub-total adrenalectomy has resulted in disappearance of a diabetic state which had existed in some instances for a number of years It should be remarked, however, that not all patients with Cushing's syndrome exhibit diabetes and conversely diabetes of Cushing's syndrome is not always reversed by adrenalectomy.

From this clinical knowledge and from the work reported with the use of adrenal steroidal compounds in animals between 1940 and 1947* it was to be anticipated that ACTH and at least some of the cortical steroids would be diabetogenic in man This was first demonstrated in a normal young man by J. S L. Browne (1943) who had administered a purified preparation of ACTH for only two days. Five years later, with greater availability of purified ACTH we undertook a study of its effects upon carbohydrate metabolism in normal humans (Conn, Louis and Wheeler, 1948, Conn, Louis and Johnston, 1948, 1949*a*) We reported the following observations (1) With large daily doses of ACTH a state of diabetes characterized by hyperglycæmia and glycosuria can be induced in normal people (2) The type of diabetes induced is relatively

*Long, Katzin and Fry, 1940, Ingle, 1940, Ingle and Thorn, 1941, Ingle, 1944, Ingle, Sheppard, Evans and Kuizenga, 1945, Ingle Sheppard, Oberle and Kuizenga, 1946, [illegible]

ADRENOCORTICAL STEROIDS ON CARBOHYDRATE METABOLISM IN MAN

JEROME W. CONN

CLINICAL investigation in man, while crucial, often leaves much to be desired from the mechanistic point of view. Man does not lend himself easily to experiments involving, for example, use of the isolated diaphragm. As a result, one can merely describe the metabolic consequences and accompaniments of a given procedure which has been applied to a human being.

The purpose of this review is to bring together what information is available regarding the effects of adrenal cortical steroids upon carbohydrate metabolism in man. For several reasons it seems important that such an evaluation be made periodically. First, it has become increasingly clear that the effects upon carbohydrate metabolism of crude anterior pituitary extract, purified growth hormone, ACTH and adrenal cortical steroids vary with the species of animal studied. Secondly, it must be borne in mind, in relation to this conference, that the initiating cause of the common variety of diabetes mellitus in man remains unknown, involving, as it does, the imponderable hereditary element. In clinical medicine we do recognize certain unusual types of diabetes as being analogous to their experimental production in animals, as, for example, in acromegaly, in Cushing's syndrome, in the presence of phæochromocytomæ following total pancreatectomy, etc. But whether or not initiating factors such as these play a rôle in the ætiology of the usual type of diabetes remains to be proven. However, once the ordinary variety of diabetes has been permanently established by whatever may be the inciting factors, it is clearly a situation then characterized by an insufficiency of peripheral insulin activity. In turn, insufficient

et al., 1950), that cortisone lessens the sensitivity to insulin of patients suffering from Addison's disease, maintains the blood sugar of such patients during prolonged fasting, and greatly intensifies hyperglycæmia, glycosuria and ketonuria of patients who have coexisting Addison's disease and diabetes mellitus. It has been suggested (Sprague *et al.*, 1949) that a diminished functional reserve of islet tissue accounts for the more intense diabetogenic activity of ACTH or cortisone in one apparently normal individual as opposed to another. Thus, Sprague *et al.* (1950) noted that two of four patients who exhibited impairment of carbohydrate tolerance under the influence of cortisone had positive family histories of diabetes. While the reserve capacity of the pancreas for insulogenesis must be important in this regard, we are somewhat perplexed at some recent observations made in our own laboratory (Fajans and Conn, unpublished). When a single injection of 100 mg of ACTH is given to mild diabetics who do not require insulin the glucose tolerance test does not change significantly from the control curve On the other hand, when the same procedure is carried out in normal individuals, with and without family histories of diabetes, a great majority exhibit significant impairment of carbohydrate tolerance. In all three groups a comparable eosinopenia is induced by the administered ACTH.

In man it has been difficult to evaluate precisely the relative diabetogenicity of the pure adrenal cortical steroids. Our own experience and that of others would indicate the following order of diabetogenic potency in apparently normal humans hydrocortisone, cortisone, corticosterone, 11-dehydrocorticosterone and 11-deoxycorticosterone. In the amounts administered in man to date 11-deoxycorticosterone has no demonstrable effect upon carbohydrate metabolism and 11-dehydrocorticosterone exerts a very mild, if significant, effect. (Conn, 1950, 1951, Conn, Fajans and Louis, 1951, Forsham *et al.*, 1946, *Sprague, personal communication*).

Because the true significance of the fall in the concentration of blood glutathione induced by ACTH, hydrocortisone and

resistant to the hypoglycæmic effect of exogenous insulin. (3) Simultaneous administration of insulin along with the ACTH does not interfere with the development of the diabetic state. (4) Although hyperglycæmia is present, diminished renal tubular reabsorption of glucose is in part responsible for the glycosuria. (5) Some normal individuals exhibit glycosuria without hyperglycæmia in response to ACTH. (6) Although a correlation exists between the degree of negative nitrogen balance and the severity of the diabetic state induced by ACTH, glyconeogenesis from protein is not accountable *per se* for production of the diabetic state. (7) It is possible that interference with peripheral utilization of glucose (decreased oxidation and/or diminished conversion to fat) is in part responsible for the production of steroid diabetes (8) A fall in the concentration of blood glutathione is associated with the induction of steroid diabetes in man. Because of the interesting triad of events occurring simultaneously under the influence of ACTH, namely, diabetes mellitus, increased uric acid excretion, and decreased concentrations of blood glutathione, it was suggested that in addition to the mechanisms indicated above, the β cells of the islets of Langerhans might be exposed to an alloxan-like intermediary with a resultant functional impairment in insulin production. (9) When assayed on the basis of adrenal ascorbic acid depletion, some batches of ACTH appear to be much more diabetogenic in man than others. This suggested that there might exist more than one type of ACTH or that the ascorbic acid depletion assay did not run parallel with all of the metabolic effects as they are observed in man.

That an anti-insulin effect in humans is exerted by ACTH and by 11-17 oxysteroids is evidenced by a number of other clinical observations. Administration of either ACTH or cortisone to patients with pre-existing diabetes intensifies the diabetic state and increases the insulin requirement significantly (Boland and Headley, 1949, Brown *et al.*, 1950; Perera *et al.*, 1949, Forsham *et al.*, 1950). It also has been observed (Sprague *et al.*, 1947, Sprague *et al.*, 1949, Forsham

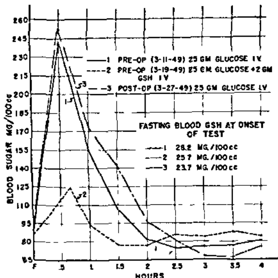


FIG. 1. Effect of intravenous reduced glutathione (GSH) upon carbohydrate tolerance in Cushing's syndrome

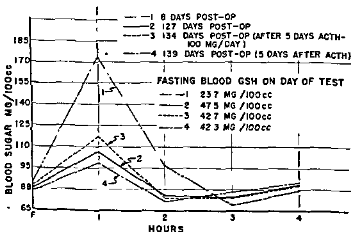


FIG. 2 Disappearance of diabetes and increase of blood glutathione (GSH) following subtotal adrenalectomy in Cushing's syndrome. (Test 25 gm. Glucose IV.)

cortisone remains obscure, the phenomenon should receive further consideration. Our group has observed that during the induction of diabetes in normal people with either ACTH or hydrocortisone the concentration of glutathione in the red blood cells diminishes (Conn, Louis and Wheeler, 1948, Conn, Louis and Johnston, 1948, 1949*a*). This observation has been confirmed in man by Hess, Kyle and Doolan (1951) but Sprague *et al.* (1950) observed no such changes. More recently it has been found (Lazarow, 1952; Goldzieher, Rawls and Goldzieher, 1952) that cortisone-induced diabetes in rats is associated with a significant fall of blood glutathione and that the sulphhydryl concentration of the tissues themselves is diminished by excessive adrenosteroidal activity. It has been reported (Conn, Louis and Johnston, 1949*b*) that intravenous administration of reduced glutathione in the course of induction of steroid diabetes in man results in a transient alleviation of the diabetic state. On the other hand Lazarow (1952) has observed intensification of cortisone-induced diabetes in rats when reduced glutathione was administered At present the divergent findings in the two species cannot be reconciled.

Whether or not the fall in blood glutathione reflects increased activity or production of an alloxan-like intermediary in the tissues remains to be established by future work. That the decreased concentration of blood glutathione does, however, appear to be related to the presence of diabetes is suggested by our further observations. Figs. 1 and 2 represent data obtained from a case of Cushing's syndrome with diabetes in which the diabetes disappeared following subtotal adrenalectomy. It will be observed in Fig. 1 that the blood glutathione was subnormal at the onset of each of the three glucose tolerance tests. Diabetes existed before operation and for a short period of time after operation However, in the preoperative period when two grams of reduced glutathione was given intravenously along with the glucose a normal curve was obtained. In Fig. 2 it is observed that by 127 days after operation the fasting blood glutathione had returned to a normal value and the subsequent glucose tolerance tests were normal



FIG 3 Patient (aged 20) with typical features of Cushing's syndrome.

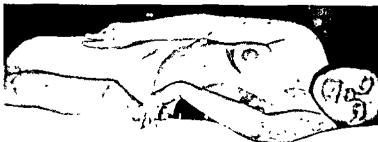


FIG. 4 Patient (aged 20) with typical features of Cushing's syndrome

[*To face page* 172

In contrast, we wish to show the findings in another case of Cushing's syndrome, *in which diabetes was not present. That* this case presented the classical picture of Cushing's syndrome is indicated by her photographs (Figs. 3 and 4). Fig. 5 shows that the level of her fasting blood sugar was consistently at the lower limits of normal and that the fasting blood gluta-

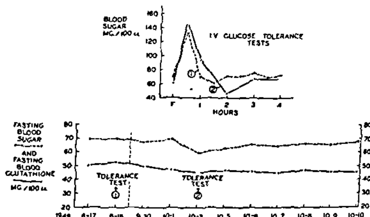


Fig 5. Cushing's syndrome with normal carbohydrate tolerance and high blood glutathione (Photograph of patient in Figs. 3 and 4.)

thione was *consistently at the upper limits of normal. It is* also to be observed that both glucose tolerance tests fall *within the normal range.*

Summary

1. When administered in large doses to man ACTH is capable of inducing a diabetic state characterized by hyperglycæmia, glycosuria, decreased renal tubular reabsorption of glucose and relative insulin resistance. It is the same type of diabetes which occurs spontaneously in Cushing's syndrome. *Some patients with classical Cushing's syndrome do not* develop diabetes. Some normal individuals given large doses of ACTH likewise do not develop diabetes.

2. Adrenal cortical steroids of the 11-oxygenated type pro-
luce a similar type of disturbance in carbohydrate metabolism
vhen administered to man. In descending order of diabeto-
genic potency they are hydrocortisone, cortisone, corticos-
erone and 11-dehydrocorticosterone.

3. Steroid diabetes in man is associated initially, at least,
vith great catabolism of body protein. However, glyconeo-
genesis from protein cannot account alone for the diabetes
which is observed. Interference with the normal pathways
or disposal of glucose appears to play a major rôle.

4. While resistance to exogenous insulin can be demon-
strated in steroid diabetes, it is not known whether the
pancreas under conditions of steroid diabetes is secreting
subnormal, normal or supernormal quantities of insulin.

5. Clinical evidence suggests that when the functional
reserve of the pancreatic insulin producing mechanism is
diminished a more intense form of diabetes is elicited in the
presence of an excess of carbohydrate active adrenal steroids.

6. ACTH, hydrocortisone, and cortisone are capable of
reducing the level of blood glutathione in man. This pheno-
menon appears to be related to the appearance of the diabetic
state. A similar relationship exists in Cushing's syndrome
with diabetes. The precise significance of this relationship
must await future investigations.

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circumstances, what kind of increase you would have in intracellular glutathione?

CONN No, I have no idea

LONG. Does it rise in the red cells significantly when you give it intravenously?

CONN: That we haven't determined All I can suggest about that is the protective effect of intravenous glutathione against alloxan diabetes

LONG Oh yes, but that may be merely using the circulation as a test-tube. Ascorbic acid will give you similar protection against alloxan diabetes

FOLLEY. Racker has recently reported, in a contribution to *Nature*, that glutathione is a sort of prosthetic group of the glyceraldehyde-3-phosphate dehydrogenase I wonder if there could be any connection there with your observations on diabetics in respect of the glutathione level in blood?

CONN I don't know.

MIRSKY I would like to draw attention to a few points which Dr Conn has made. First, I want to emphasize that the type of diabetes produced by steroids differs markedly from the so-called spontaneous form of clinical diabetes mellitus The difference is very clearly demonstrable. Secondly, there was the interesting observation that the glucose tolerance curve of the patient with mild diabetes was not further decreased with the administration of cortisone We had occasion to study a number of subjects who were selected because they had had diabetes in the past and no longer have it These were subjects with real potential diabetes, because they had had diabetes on one or two occasions in the past which had lasted for more than two years The administered ACTH in these subjects did not produce any abnormality. Further, administration of cortisone to patients with ketosis may actually reduce the ketosis We are dealing with different clinical situations The status of a patient may determine the responsiveness

BEST I'm trying to think of someone who has studied the effect of insulin in combating the aggravation of diabetes by ACTH in animals

CONN: It has been done with crude extract, but I'm not aware of any study which has been presented with ACTH

WILHELMI I'd like to ask Dr Conn about the ACTH preparations that he used The point which interested me particularly is that the

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H., and POLLEY,

DISCUSSION

As
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administered I recall the work of

think we are all in agreement that there is tremendous variability among diabetics as to whether or not increased insulin will be necessary when cortisone is administered There are some diabetics who require a very significant increase, perhaps 5 times the base-line level, while others appear not to need very much more insulin What the explanation is, I don't know.

LAWRENCE: Can you tell me please, in that respect, whether those who need a great increase are much milder than the severe ones? I ask this because in my experience—not large—in treating severe diabetics with cortisone for rheumatoid conditions (by severe cases I mean people needing 60-100 units of insulin per day) there has been no increase at all in the insulin requirements I just wondered if this discrepancy reported is perhaps related in any way to the severity or the actual nature of the diabetes?

CONN: We haven't seen any correlation between severity and the need for more insulin under cortisone therapy The only thing that we have noticed is that in mild diabetics who maintain a normal fasting blood sugar without insulin, there is frequently no exacerbation of the diabetes from cortisone. On the other hand we have observed the production of virtually complete insulin-resistance in a 75-unit diabetic given large doses of ACTH.

LUKENS Perhaps I can answer your question in part, Dr Lawrence, by supplementing your experience with our three diabetics with arthritis One of them needed no increase in insulin requirements, the others had to have their dose of insulin approximately doubled All three would be regarded as severe diabetics in the sense that they needed 60–100 units of insulin per day

SWYER Dr Conn pointed out that gluconeogenesis cannot adequately account for the diabetogenic action of corticoids and that there must be interference with the peripheral utilization of carbohydrates. Perhaps lipogenesis might be interfered with. The obesity, which is the common accompaniment of the Cushing's syndrome, doesn't seem to fit in with this idea, and I wondered what Dr Conn has to say about that?

CONN. Well, I have an idea about it First the obesity exhibited in Cushing's syndrome is related more to redistribution of fat than to the laying down of new fat Secondly, if lipogenesis is impeded by an excess of 11-oxysteroid activity, this inhibition of lipogenesis may not be a complete one at all and very likely isn't

SWYER In other words, the inhibition of lipogenesis is relatively a minor thing?

CONN Yes, it may or may not be, depending on the circumstances Insulin is required for lipogenesis but many mild diabetics not taking insulin grow fat

BEST Quite a striking effect of both growth hormone and of insulin

diabetogenic action of some preparations are not very easily correlated with the adrenal ascorbic acid depressant effects. With the early protein ACTHs, a very real question is the possible contamination of these preparations with growth hormone. There is no assay available which will allow one to detect reasonable quantities of growth hormone in the presence of an overwhelming surplus of ACTH That raises the possibility that the diabetogenic potency of present ACTH preparations may be more nearly correlated with the order of contamination with growth hormone. It raises also the possibility that with more recent ACTH preparations of the Astwood type, which may be simple peptides relatively free from growth hormone after the treatment with oxycellulose, the diabetogenic activity will be appreciably less in relation to the adrenal ascorbic effects

CONN: The preparations which we used were not diabetogenic or

re were

should

REID I'd like to ask Dr

Wilhelmi said In testing

pH was the injection giver

crude preparation of "corticotropin," obtained from beef glands by

may be differences simply in absorption rates, which might be eliminated by administering the preparations as tannates, for example.

CONN I don't know about possible differences in absorption rates. With respect to the first question, the ACTH was given in alkaline solution to dogs. It was not an Astwood type of preparation.

MACH As Dr Conn just mentioned, it must be emphasized that there

diabetes through cortisone administration

I had a diabetic patient who required 15 units of insulin before he was treated for a myeloma, the injection of 100 mg of cortisone aggravated the diabetes to the extent that 90 units of insulin were necessary to prevent hyperglycæmia However, in another diabetic patient who also had to receive 100 mg. of cortisone, it was sufficient to increase the dosage of insulin by one-third only to obtain a normal glycæmia. It is not exact to say that diabetes caused or aggravated by cortisone is resistant to insulin.

We have also demonstrated a low glucose-tolerance curve in a patient with pan-hypopituitarism. On treatment with ACTH, the curve gradually rose, showing after 4 weeks a marked increase in the amplitude of the curve. Have you observed that hyperglycæmia may

CLINICAL OBSERVATIONS ON METABOLISM IN OBESITY

H W. BANSI

FAT is the essential storage substance in energy exchange, and in contrast to glycogen and proteins, it can be stored up in the body in any quantity. Nevertheless, once growth has ended, it is one of the fundamental laws of life that the weight of the body must be kept constant—no matter what energy is necessarily expended from time to time—by the automatic regulation of the food intake on a freely chosen diet. There is a subtle distinction between "Fettsucht" (obesity), as the expression of a certain lipophil tendency of the body, and "Fettleibigkeit" (corpulence), which signifies the state of supernormal fatness

It has long been known that fatty tissue is not an inert substance taking no part in metabolism, and this has been conclusively proved by isotopic research. The fact that only about 400 g of glycogen are contained in the body of a man weighing roughly 70 kg., and that this quantity—if no more calories were provided by the food—would be entirely exhausted after 24 hours at the most, shows that fat must be continually mobilized in the cycle of metabolic processes.

Obesity is certainly not a single clinical entity. It may appear in numerous endocrine diseases, for example, Cushing's disease, the obese type of diabetes and several cerebral—particularly hypothalamic—dysfunctions, whereas the participation of the pituitary itself, which was formerly generally assumed in clinical medicine, is to-day once more the subject of controversy.

Little is yet known about how the intermediate fat metabolism takes place and in particular where—only in the liver, or in the periphery? This applies particularly to the almost

is the increase in appetite. If you give either to animals, they may eat two or three times as much.

LAWRENCE: There is just one more point on which I would like some information and that is on the glutathione question. Have you found it increases in what we might call ordinary diabetes?

CONN: No, we haven't.

LAWRENCE: Except in ketosis, do you find it there?

CONN: We haven't studied it.

LAWRENCE: Well, it goes down quite markedly in the severe ketotic state.

CONN: That is very interesting, because as I mentioned yesterday, 11-oxy-steroids rise sharply in the presence of ketosis.

HOET: I gave 50–100 mg of cortisone to patients with Addison's disease, and five developed diabetes with a blood sugar higher than 200 mg per cent even if they started with a blood sugar of 40 or 50. Everybody knows now that in a case of Addison's disease you must never give more than 20–40 mg of cortisone. If you give 100 mg. or 150 mg./day you get the blood sugar increased, diabetes, polyuria, etc. The adaptation of the pancreas to any secretion of cortisone is disturbed.

MIRSKY: We have studied women who gave birth to babies weighing 10 lb or more, and in whom the incidence of diabetes in the future can be predicted to be approximately 40 per cent. We attempted to find whether through the administration of a provocative dose of ACTH [illegible] We [illegible]

MIRSKY: 50 mg

GRAY: Would not that show even more definitely that natural [illegible] diabetes?

[illegible] ide-

[illegible] nals were given *stress* of various types

MIRSKY: Rats not dogs——

CONN: Animals!

MIRSKY: I think it is a very important point and the distinction has to be made, because it is an old observation that dogs do not show a drop in the blood sugar.

obese patient, we found an average glycogen content of 7·2 mg. per cent in the fresh substance, and of 8·9 mg. per cent in the dry substance, with an average water content of 23·0 per cent. Since the average content in the serum is 3 mg. per cent, it is seen that the principal share of the glycogen content falls to the fatty cell, and not to the extracellular water. The

Table I

GLYCOGEN AND WATER CONTENT IN BIOPSY OF FAT TISSUE AFTER A TOTAL STARVATION PERIOD OF SIX TO TEN DAYS, IMMEDIATELY BEFORE THE OPERATION. PATIENT RECEIVED CARBOHYDRATE AND INSULIN.

Patient	Carbohydrate (g.)	Insulin (units)	Glycogen mg.% Fresh fat	Glycogen mg.% Dried fat	Water content %
Se.	100	20	9.3	-	-
Scho.	250	30	13.4	18.7	28.30
St.	250	16	2.0	2.6	22.89
K.	259	20	6.3	7.8	19.03
So.	259	48	8.7	10.6	18.00
Bl.	259	50	6.4	8.0	21.04
Ba	285	52	4.0	5.6	28.46

further identification of the polysaccharide, for example its change into phenylhexosazone, and the determination of its melting-point, has so far not been completed.

The comparative study of the sugar contents of capillary and venous blood provides some clue to peripheral sugar metabolism. The loss of glucose to the tissue through which the blood flows corresponds to the difference between the sugar contents of the two kinds of blood The prerequisites, such

lipomatous-looking localization of the fat deposits which appears frequently in clinical medicine (panniculitis, lipomatosis-like rolls of fat).

We shall not discuss here which hormones, alone or in conjunction with the autonomic nervous system, are concerned in any one case. I shall restrict my remarks to observations which concern protein metabolism during fat formation and fat reduction, also to peripheral glucose metabolism, in the hope of getting some insight into the active or passive processes at work in the periphery in adiposity.

Numerous investigations on animals by Wertheimer (1927), Hausberger (1938), Engel and Scott (1950) and other authors have proved that the direct accumulation of carbohydrates in the peripheral fatty tissue also plays a part in the production of fat To my knowledge this has as yet only been proved in isolated cases in the human being (Arndt, 1926). Our earlier attempts to demonstrate glycogen in the fatty tissue in obese patients when large quantities of fat were removed surgically always proved unsuccessful, even when glucose was previously administered Only when, prompted by the findings of Engel and Scott, after a long period of fasting we administered insulin simultaneously with large quantities of carbohydrates (not only in the form of glucose, but also as rice, to ensure a slower passage into the blood stream) did my collaborators Backhaus and Fretwurst and I succeed in obtaining glycogen in the abdominal fat of extremely overweight patients. The abdominal fat here does not actually represent an ideal "fat organ" with a lively intermediate metabolism, like, for example, the "brown fat organ" of the rat

In view of the difficulty of a specific demonstration of this polysaccharide we used the method of Pfluger which is still in current practice. In this method of analytical demonstration, glycogen is defined as the white, amorphous substance which, resistant to hot 60 per cent solution of caustic potash, is extracted from the tissue and reduced after acid hydrolysis, and is quantitatively determinable by its reducing capacity.

Using this procedure to examine the abdominal fat of the

obese patient, we found an average glycogen content of 7·2 mg. per cent in the fresh substance, and of 8·9 mg. per cent in the dry substance, with an average water content of 23·0 per cent. Since the average content in the serum is 3 mg. per cent, it is seen that the principal share of the glycogen content falls to the fatty cell, and not to the extracellular water. The

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The comparative study of the sugar contents of capillary and venous blood provides some clue to peripheral sugar metabolism. The loss of glucose to the tissue through which the blood flows corresponds to the difference between the sugar contents of the two kinds of blood. The prerequisites, such

as a preceding period of starvation, etc., certainly have an effect on sugar metabolism in the peripheral tissues. Therefore a degree of caution is necessary in drawing conclusions from the difference between the arterial and venous blood sugar. In the literature the arterio-venous difference after administration of sugar is given as approximately 14 mg. per cent. The arterio-venous difference in blood sugar was greater than normal, particularly in those patients with lipophil dystrophy

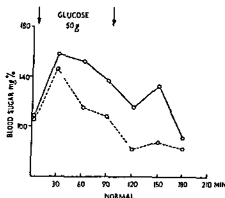


Fig. 1. Capillary-venous differences of blood-sugar in a normal case after two successive doses of 50 gm of glucose (Staub-Traugott).

who were in a stage of abnormally active fatty "disease" (Mangelfettsucht—Bansi, 1947). Several sugar curves following double-loading of very severe cases of lipophil disease during the feeding-up stage are seen in Figs. 1 and 2 Here the peripheral sugar consumption is on occasions so heavy that the venous blood curve is practically a straight line—*in other words, all the blood sugar passing the liver* (or in Soskin's view, all the sugar supplied from the liver) is continually being stored at the periphery. At the same time Gulzow (1947) made similar observations independently of us in cases of starvation during the feeding-up stage, whereas in starvation which is not yet compensated he saw the syndrome

of "starvation diabetes," namely an abnormally slight removal of sugar from the periphery.

This behaviour is, however, typical only of most cases of lipophil dystrophy, whereas most other adipose patients, whose obesity is already established, show no such striking anomalies. Occasionally the arterio-venous difference reaches 30 mg. per cent and more, but on the whole does not remain above normal. Sometimes a raised blood sugar consumption

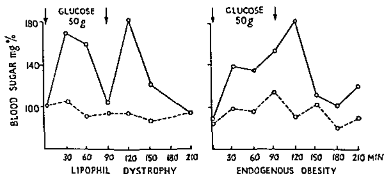


FIG. 2 Capillary-venous difference of blood-sugars after two successive doses of 50 gm of glucose in cases of lipophil dystrophy and endogenous obesity (Staub-Traugott)

was also seen in the second phase, an observation which might perhaps best be interpreted in the old sense as an increased insulin output, which is accompanied by an increased production of fat in the periphery [here I must refrain from introducing into the discussion the question of the homeostatic mechanism of the liver as conceived by Soskin (Soskin and Levine, 1946). The tendency of the periphery to retain a considerable quantity of sugar remains undisputed But the blood sugar curves are certainly dependent upon many factors. Of course the patients were kept in a state of absolute muscular rest for the whole duration of the experiments]

That the increased sugar removal is seen almost exclusively in dystrophic patients in the pronounced stage of lipophilia

may perhaps be attributed to the fact that in already overweight patients the practically continuous storing up of glycogen and attendant fat formation is no longer so pronounced, more particularly as the parts of the tissue which are usually involved in blood sugar analysis show no abnormal tendency to store fat. Tests using blood from the veins of fat thighs also yielded no very great arterio-venous differences, probably because they really took in only the superficial circulation of the limb.

These indications of glycogen formation in the peripheral fatty tissue, and the sometimes remarkably heavy loss of sugar from the blood at the periphery seem to me to show a considerable avidity for sugar at the periphery, and even suggest new fat formation in the fatty tissue.

We were also interested in the protein metabolism in various overweight patients. Albright, Parson and Bloomberg (1941) were the first to draw attention to the fact that in Cushing's disease a catabolic state of metabolism may exist in respect of the protein economy, and this we were able to confirm in various cases. During the years of shortage in Germany, and especially in soldiers returning from long captivity, we noticed a peculiar disease during the feeding-up phase, which I have termed lipophil dystrophy (Mangelfettsucht) and which could be followed up in thousands of repatriated men. In this metabolic state, which is associated with a diet relatively rich in carbohydrates but generally very poor in protein, and in which

while affected with

of manifest œdema,

e in a very remarkable way, and numerous metabolic-balance tests repeatedly confirmed this. On a diet of sufficient calorie value to maintain life, with the normally adequate protein intake of 70–80 gm. (up to a third or half in the form of animal protein), although there was a rapid increase in weight, it was not possible in either these men or in women who had been undernourished for a correspondingly long period, to obtain even a steady nitrogen balance, let alone the positive one which is usual

after a fairly long period of undernourishment or in convalescence. During this development of lipophil dystrophy, the patients showed numerous other, predominantly vegetative disturbances, also often a swelling of the parotid gland and gastro-intestinal symptoms, such as meteorism, relative enzyme deficiency, impotence, gynæcomastia and others besides, as I have described in detail in my monograph on starvation œdema (1949)

In genuine deposition of fat proved by tissue excision and a weight increase that is only to a very slight degree

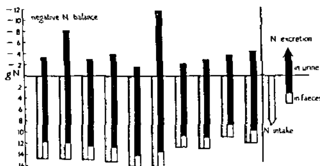


FIG 3 Nitrogen balance in four cases of lipophil dystrophy

attributable to water retention, the *protein balance on a normal diet was more or less negative* For the purpose of analysing this disturbance of protein metabolism more closely, the *protein minimum* was investigated in a large number of these patients with very careful observations of metabolism—including the loss of nitrogen in the stool—in order to determine so to speak the wear and tear quota of the body, that is, the lowest point in endogenous protein metabolism With a caloric intake of 2,500 in a diet containing practically no protein (0 3 g. of nitrogen), the nitrogen loss was essentially higher (50–110 mg.) than the normal nitrogen elimination of 30–50 mg. per kg. of body weight as given by various authorities. The nitrogen turnover in these obese patients therefore—for here we were

concerned with one phase of fat accumulation—was absolutely catabolic. A resemblance will be noted between this condition and a disturbance of those hormones which primarily promote nitrogen production (testosterone deficiency or over production of the S-hormones of the adrenals), in the course of which at least small quantities of fat are formed from protein. In cases with a negative nitrogen balance, my collaborator Lohmeyer and Anhelm (1951) found an increase in 17-ketosteroid elimination, which might be regarded as supporting a disturbance of pituitary function on the lines of Cushing's disease; at the same time an increase in the adrenocorticotropic anterior pituitary hormone also explains the hypertrophy of the adrenals which is observed in malnutrition. Unfortunately we could not determine the oxycorticosteroids, which would have made the picture more complete.

The obese patient of the pituitary-adrenal-type, in the sense of *partial overactivity* of the adrenal cortex, therefore tends to have an irrational protein metabolism. In this he is quite different from the majority of normal adipose patients, whose protein metabolism will be described now.

Normally in clinical medicine there is little opportunity for following the development of corpulence. Apart from some cases of weight increase of 10–20 kg. occurring in the course of a few weeks, it generally takes several years to develop. Hence the patient only seeks the advice of a doctor after he has become considerably overweight. It seemed therefore practical and sensible to study the *process of losing weight* within the framework of the general metabolism, especially as it must be the object of every weight-reducing treatment, when cutting down the calorie intake, to spare the protoplasm components which are not increased, and to reduce only the fat. Two basic problems presented themselves: (1) How does the protein metabolism of adipose patients behave when the calorie intake is cut down to about 800, but enough protein to maintain life—about 70 g.—is given, and (2) how does loss of substance take place where food is practically withheld altogether—as in treatments with fruit juice and starvation

So far only very isolated investigations of these problems exist.

In the course of metabolic-balance tests extending over several weeks, my collaborators and I (Bansi *et al.*, 1951, 1952) examined altogether 76 cases of obesity of different types—but not including patients with lipophil dystrophy—all considerably overweight. The findings were as follows:—

With a restriction of the total calories to 800, but a daily intake of 10 g. of nitrogen in the form of first-class animal protein, the majority of overweight patients, after a brief adjustment of their nitrogen economy, show a steady or even slightly negative nitrogen balance, and in only two of these did the daily losses of nitrogen exceed 2 g. The patients concerned were young girls with a Cushing-type obesity and markedly increased 17-ketosteroid elimination. In some patients for whom we prescribed the insertion of a period on fruit juice, the periodic alternation of the two forms of diet soon produced a steady nitrogen balance during the 800 calorie periods. The body therefore adapts itself to the deficiency of calories, and may after a time set in motion the mobilization of fat, which is believed to be so difficult in adiposity, so that it can cover its energy requirements to a great extent out of its fat deposits.

This form of diet is therefore suited to the reduction of the fat deposits, without jeopardizing the protoplasm store of the body.

Actual starvation metabolism also behaves in a remarkable way in most adipose patients. In these tests the protein intake was practically nil. In pure starvation tests, although fat is still present in the depots, and the majority of the caloric needs are met by burning up the fat reserves, nevertheless more protein is used up in this state of affairs for the purpose of energy than corresponds to the wear and tear quota, that is, to the endogenous nitrogen minimum. In Fig. 4 the protein destruction (in g. of nitrogen) was correlated with diminishing calorie intake in a graph. In making this, we used the starvation tests published in the literature, and a number of our

own metabolic-balance tests on a protein-free diet and with a diminishing intake of calories. It is seen that the nitrogen consumption only becomes distinctly higher than what corresponds to the wear and tear quota when the intake is reduced to below 800 calories, and then, when the calories are reduced to under 400, it rises sharply. The quantity of nitrogen is

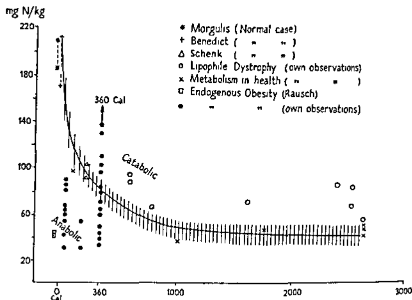


Fig. 4 Nitrogen excretion in mg /kg with protein-free diet and ascending calorie intake.

calculated per kg. body-weight. In one series of tests about 380 calories were given daily in the form of fruit juice, while a second series was subjected to complete starvation, which most of the patients readily tolerated for 10 days.

Among 31 cases on the fruit juice diet, a nitrogen elimination of between 2 4 and 8·36 g., or between 34·8 and 134 mg. per kg. actual weight, was demonstrated. Fifteen cases lay within the normal limits of 55–100 mg./kg., and only two above the normal, which two therefore showed an increased nitrogen

destruction during the starvation phase, while 14 patients showed a clearly reduced nitrogen metabolism during this period of reduced food. The quantities of nitrogen eliminated during these periods corresponded to those of the "normal" wear and tear quota, determined by Thomas' (1910) process in normal human beings after 3–5 days on a diet which is sufficient in calories, but free from nitrogen.

The economical nitrogen balance is observed just as clearly, if not more so, in strict fasting, even if, as a result of the complete cessation of calorie intake, a somewhat greater elimination of nitrogen is present. In complete fasting the daily loss of nitrogen in professional starvers and normal people is between 150 and 220 mg./kg. body weight. In our 9 cases of endogenous obesity without Cushing's symptoms—in tests lasting 10 days—the elimination of nitrogen after 2–3 days calculated from several days' averages was between 39·5 and 64·4 mg./kg. body weight, in other words, nearly the same as the endogenous protein minimum. The majority of the adipose patients behaved therefore, even with a prolonged period of fasting, as if they were receiving a diet free from protein only, but containing sufficient calories, and broke down protein only in accordance with their internal economy. It remains a very remarkable finding, in respect of the

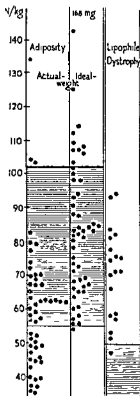


FIG. 5. Nitrogen excretion (mg./kg./body-weight) during a protein-free diet of 360 calories in cases of endogenous obesity (adiposity), and a protein-free diet of 2,300 calories in cases of lipophil dystrophy. The grey districts represent the limits of normal variation.

practicability of drastic calorie restriction, of the breaking down of fat, and of the switch to an economical metabolism. We do not dare to indulge in speculations on the mechanisms at work. We shall merely put the following questions for discussion—

(1) Is the exceedingly economical and endogenous metabolism of nitrogen, which is present in obese patients—apart from those with lipophil dystrophy and the Cushing type of obesity—the expression of a general economy in metabolism and equivalent to a tendency to create fat reserves, or

(2) Is the economical nitrogen metabolism only a sign that the fat reserves can easily be made available by the body to cover its energy needs, which is contrary to the view held hitherto, that they are not easily broken down in adiposity.

The protein metabolism in adiposity therefore behaves as follows.—

Only in lipophil dystrophy and the Cushing types, which both show signs of adrenal hyperfunction, is the protein economy catabolic and tending towards an increased protein destruction (perhaps for liponeogenesis). The majority of overweight patients show an extremely slight endogenous protein turnover, and obesity can be reduced satisfactorily by restricting the calorie intake If they are guaranteed an adequate supply of protein, then they only draw on their reserves of fat, and spare the deposits of protoplasm more readily than the normal person does on the same food. This is particularly true of a protein-free diet, where the losses of nitrogen on a diet giving about 400 calories correspond, in over 50 per cent of the patients, to the endogenous protein minimum, and with complete fasting exceed this only slightly. The fat destruction is therefore a straightforward process. Whether there is a pathogenic connection between this abnormally frugal protein metabolism and the accumulation of fat is a debatable question.

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DISCUSSION

BEST Does that mean that you can change the condition by just substituting a high carbohydrate diet for a high protein diet?

BANSI Yes

BEST Can that be reversed?

BANSI No. It is a feature of a person who has a high carbohydrate intake with a very low protein intake I could have shown you another picture, but I will not take your time This was a typical case of the person who after a short period of starvation gets a huge intake of potatoes and bread

BEST Did you have any liver biopsy?

BANSI No.

SHERLOCK We did a group of liver biopsies on under-nourished

diet

MANN Is there any connection between the forms of malnutrition described by you and some forms of hunger œdema?

BANSI Yes, or at least in the reparation stage of malnutrition

SWYER These patients are under a form of "stress" in the sense that Selye uses it, are they not? Physical stress from the sudden change of dietary habits?

BANSI Yes, and probably there might also be a type of psychological stress There are also other cases where you get it in camps

SWYER There must be an increased calorie quota to put on weight?

BANSI The calories must be augmented

SWYER That in itself would very likely throw a certain amount of

stress on the organism as a whole, stress which would activate the adrenal cortex.

BANSI I believe the quantity of calories was not very high. It could not go above 3000. They had a low B.M.R. and were accustomed to a reduced food intake.

SWYER Precisely, and therefore this sudden change to a higher food intake would constitute a stress.

BANSI They were very nervous——

BEST May I suggest that we define the word "stress" as we use it If we do that, we may not need to use it at all

HOET I would just ask if you saw a good deal of glycosuria in those patients in the camps? I saw a dozen cases which had glycosuria for 7 days when they came back, and their hyperglycæmic curve was going up to 200 mg. per cent, then it recovered. One saw that on the very first days in the sons of medical men, for example, because tests for sugar in their urine has been made early, i e. as soon as they came back If you see them a week or a fortnight later, you don't catch that phenomenon.

BANSI You are quite right, that is so called "hunger" diabetes It is always seen, but I did not have the patients when they came directly from the camps. Then they had also, as mentioned by Gulzow, typical diabetic hyperglycæmic curves

PART IV

HORMONAL CONTROL OF THE STORAGE OF GLYCOGEN

HORMONAL CONTROL OF GLYCOGEN STORAGE

*JANE A. RUSSELL**

The principal form of carbohydrate in animal tissues, glycogen, is found there in amounts which are small compared to the energy needs of the organism. It would be expected then that this glycogen would usually be undergoing rather rapid utilization and replacement At present, data on the rates of glycogen turnover are few and unsatisfactory, even for the tissues of normal animals Most of our conclusions about regulation of processes concerned in this turnover must then be inferred from changes in the quantities of glycogen present in the tissues in different circumstances.

The amount of glycogen present in a tissue at any time must of course be the resultant of the rates of many processes: of absorption of glucose into the cells, of its phosphorylation, and of dissimilation and oxidation of the carbohydrate to other substances. The rates of these processes in turn may be much affected by other metabolic events, such as by changes in the effective concentrations of intermediates like phosphate or ATP. It is a somewhat hazardous undertaking to try to assess the rôles of the hormones in carbohydrate metabolism from observations of their effects on the glycogen levels of the tissues, but it is worth the attempt for two reasons, the knowledge to be gained may be important in understanding the

*Supported in part by a grant-in-aid from the American Cancer Society, on recommendation of the Committee on Growth of the National Research Council

metabolic alterations characteristic of imbalances in hormonal control; and perhaps more importantly, these considerations may be expected to offer some clues to the mechanisms by which the hormones may affect intermediary metabolism

The liver is of course the site of the principal labile store of carbohydrate in the body and ordinarily this organ contains the highest concentrations of glycogen. The amounts present in the liver are, however, extremely variable. The chief determinant of the quantity of glycogen present in the liver at any time is the amount of carbohydrate available, either from the diet or formed *in situ* from other substances such as amino acids or lactate. Although the amounts of glycogen in the liver may be affected by excess or deficiency of certain hormones, these changes usually are small compared to those which may be induced independently of hormonal control by purely logistic means Also, the possible sources and ultimate fates of the carbohydrate in the liver are more varied than in other tissues. Hence, except in a few instances, it has been even more difficult to evaluate the rôles of the hormones in glycogen formation and utilization in the liver than in other tissues. For these reasons, and also because of limitations of time, glycogen formation in liver will not be further considered here.

The other principal sites of carbohydrate storage are the muscles Little is known concerning smooth muscle, but several examples of striated muscle and also the heart have been investigated extensively. In these, glycogen probably has its greatest significance as a possible source of anaerobic energy, but it must also usually be utilized oxidatively As far as is known, the only source of glycogen in muscle is the glucose of the blood. Unlike the glycogen of the liver, that of most muscles exhibits a considerable degree of uniformity during the usual cycles of feeding and fasting Data on the turnover rates of this glycogen are lacking, it would be of much interest to know whether the glycogen is really rather inert, or, if, as seems more likely, it is being formed and broken down constantly but at rates which are relatively unaffected

by the blood sugar level. In this relative independence of nutrition, the muscles would seem to offer a preferable site for investigations of the hormonal control of glycogen formation and utilization.

Before hormonal effects are considered, two points must be made in connection with the glycogen of the muscles. One is that although the glycogen content of individual muscles may be relatively constant, that of different muscles tends to differ in characteristic fashion Further, the various muscles may respond quantitatively in different degree to changes in physiological status, and in some instances (as in the heart) there may be qualitative divergence in response as well Nothing is known concerning the determinants of these characteristics; it may be supposed that eventual knowledge concerning these factors would contribute also in some degree to our understanding of the control of carbohydrate metabolism.

Secondly, it must be pointed out that the glycogen of the tissues is not homogeneous, in a physiological sense at least. In the past, some distinctions in physical properties have been made between certain fractions of the glycogen Recently, Bloom *et al.* (1951) have found that there is a large and consistent difference between the amounts of glycogen which may be extracted from fresh muscle tissues by trichloroacetic acid (TCA) and that which is found after alkali digestion. In several tissues and in a variety of circumstances (hormone treatment, fasting, glucose feeding, tetanic stimulation) the TCA-extractable glycogen has been found to be the "mobile" fraction, whereas the non-extractable glycogen remains relatively constant. Some examples of these differences will be presented later. Little is known concerning any chemical differences between these two glycogen fractions which behave so differently physiologically. In future work on the effects of the hormones on glycogen stores, a true picture of the magnitude of the changes induced will require determination of at least these two fractions of the glycogen found in the tissues.

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insulin than the other tissues In fact, in the fasting rat in which reflex epinephrine secretion has been prevented by prior adrenodemedullation, as little as 0·1 unit per kg. of insulin will double the extractable glycogen of this organ. In view of the convenience of the isolated rat diaphragm as a subject for experiment, this susceptibility to insulin has

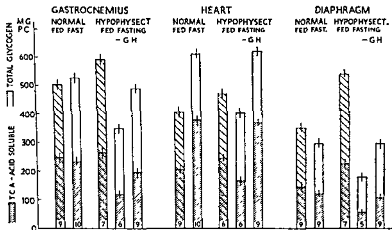


FIG 1 Effects of insulin (5 units/kg) and growth hormone (10 mg./kg) on the total trichloracetic acid soluble, and in- [illegible]

perhaps been fortunate, but it makes necessary some reservations about quantitative extension of observations made on the diaphragm to the whole organism.

Besides favouring the deposition of carbohydrate as glycogen, insulin also enhances the further conversions of carbohydrates, as has been shown by balance studies in isolated tissues and by isotope experiments in intact animals. The question then is how does insulin act—to promote the entry of glucose into the cells, to enhance the rate of the hexokinase reaction, or to accelerate some further stage of carbohydrate

Of the hormones which may affect muscle glycogen, the most studied have been insulin and epinephrine. As is well known, one at least of the effects of the latter hormone is the promotion of anaerobic glycogenolysis in muscle. Recently, Sutherland and Cori (1951) have presented evidence to indicate that in the liver the effect of epinephrine and of the pancreatic glycogenolytic agent is to increase the amount of phosphorylase in the active form. Similar studies regarding glycogenolysis in muscle are not yet available. Since the reaction catalysed by phosphorylase is readily reversible, it is not clear, if this is the mechanism in muscle, why the action of epinephrine should be always to accelerate glycogenolysis. It may be that the effective concentrations of intermediates usually are such as to favour the breakdown of glycogen but that this process goes only slowly in normal tissues because the enzyme is mainly in an inactive form. This does not seem likely, because glycogenolysis may be induced in muscle in conditions in which otherwise glycogen deposition would be occurring, as in the presence of insulin. Another possibility is that in the intact cell there are structural limitations on the accessibility of enzymes to substrates, or in other words, that equilibrium conditions do not prevail. The hormones, which at present seem always to require the intact cell for demonstration of their activities, may act in part upon the organization of these cellular systems.

Regarding the action of insulin on glycogen formation, there has now accumulated a considerable body of evidence, much of it of recent years from observations on the isolated diaphragm. The consensus from these data is that insulin promotes glycogen formation in muscle if there is sufficient glucose available to make this apparent. As shown in Fig 1, when glucose and insulin are given to the normal rat, deposition of glycogen is promoted in the gastrocnemius muscle, heart and diaphragm, in increasing order of response. As indicated earlier, most of the effect is seen in the TCA-extractable fraction of the glycogen, the remainder varying little. It may be remarked that the diaphragm is more sensitive to

is accelerated normally in such preparations. Since the increased rate of disappearance of carbohydrate and the high R.Q. continue to be seen in eviscerated hypophysectomized animals, failure of gluconeogenesis cannot account for all of the effect. Evidently, then, the utilization of carbohydrate during fasting, either by oxidation or by fat formation, must be increased in the absence of the pituitary hormones.

The loss of muscle glycogen and the elevation of the R.Q.

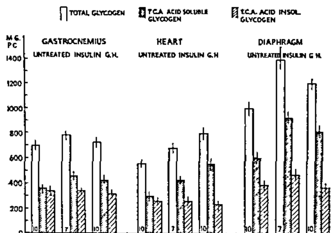


FIG 2 Effects of hypophysectomy and growth hormone on the total and trichloroacetic acid soluble glycogen of the tissue of rats during fasting The animals were fasted twenty-four hours either without treatment or with growth hormone (5 mg./kg.) given as divided doses during the fast The control animals were unfasted

in the hypophysectomized rat may be prevented completely by treatment during the fasting period with pituitary extracts (Russell, 1938*b*). Recently it has been shown that highly purified growth hormone also is able to prevent the loss of glycogen from skeletal muscle (Russell and Wilhelmi, 1950) and from diaphragm and to increase the glycogen of the heart to the normal fasting level. The figures presented in Fig. 2 were obtained after the administration of 0·5 mg.

metabolism? It has been argued that since the phosphorylation of glucose is an essentially irreversible reaction, its rate must govern the rates of all following reactions in the metabolism of the glucose so taken up, and that the action of insulin must then be on this step. This line of reasoning may be too simple, however, if as mentioned above, equilibrium conditions are not achieved by other reactions within the system. Further, there is evidence that in the absence of insulin there are metabolic defects in the metabolism of pyruvate and acetate as well as of glucose, and that added insulin may increase the oxidation of some non-carbohydrate intermediaries. It is possible, perhaps, that the increased uptake of glucose into the cells and its conversion to glycogen under the influence of insulin could be explained by effects of the hormone on the provision of energy in appropriate form and place. It may be recalled that in the diabetic animal the performance of work by the muscles also promotes the uptake of glucose from the blood; perhaps this is an example of the same process, although of course it need not be wholly identical in mechanism with the action of insulin.

We may next consider the influence of the hormones of the anterior pituitary on the glycogen of the tissues. As was shown some years ago (Russell and Bennett, 1937; Russell, 1938*a*), the hypophysectomized rat when fed maintains normal carbohydrate levels in blood and tissues, but in fasts of even rather short duration, first its liver and then its muscles become depleted of glycogen. As may be seen in Fig. 2, the heart, which normally tends to increase its glycogen content in fasting, instead also is depleted, and the diaphragm too loses more glycogen than it does in the normal fasting animal. These changes also occur mainly in the TCA-extractable fraction of the glycogen.

At the same time that carbohydrate is lost from the body, the R.Q. is elevated, and the increase in this ratio is of such an order that the disappearance of carbohydrate could be accounted for by a net increase in the oxidation of carbohydrate. There is no evidence that anaerobic glycogenolysis

carbohydrate is fed to hypophysectomized animals, it disappears from the body at something more than twice the rate seen in normal animals, despite the low metabolic rates of these animals (Russell, 1938*c*). Again the R.Q. is high. It is not known at present whether the rapid disappearance of carbohydrate is due to increased oxidation or to fat formation in excess of its utilization, but there is some evidence to indicate that the latter may be important. The administration of pituitary extract or of purified growth hormone to normal animals before the feeding results in increased deposition of glycogen and lowering of the R.Q. (Russell, 1938*a*; Milman and Russell, 1950, Illingworth and Russell, 1951). As shown in Fig. 1, the effect on the glycogen of the heart is particularly striking; for normally on glucose feeding there is little further deposition of glycogen in this organ.

A peculiar feature of this effect of the pituitary factor on the metabolism of fed carbohydrate is that it requires the presence of some adrenocortical hormone for its expression (Russell, 1940). Fig 3 indicates observations made in adrenalectomized rats given pituitary extract with and without adrenocortical extract. Similar data regarding the deposition of glycogen in the heart and diaphragm have been obtained with purified growth hormone (Illingworth and Russell, 1951). Since adrenocortical activity is not required for the action of growth hormone on the maintenance of muscle glycogen during fasting but is needed for what appear to be substantially similar activities in the fed animal, the suggestion might be that the cortical hormone is in this connection concerned with the rapid transfer of glucose into the tissues

The observations on effects of pituitary hormones so far described have been obtained in intact animals, and from these few conclusions may be drawn as to the precise site of action of the hormonal factors. In recent years some attempts have been made to define this action more clearly by studies on the isolated diaphragm This work has been summarized recently by Krahl (1951) Almost all of the observations reported to date have concerned the uptake of glucose

of growth hormone per 100 g. body weight, but smaller doses also are effective. The liver, from which normally most of the glycogen disappears in 24 hours in any case, is little affected by the hormone.

In these respects, neither ACTH nor adrenocortical extracts completely duplicates the effects of growth hormone. If sufficient quantities of the adrenal factors are given over a period of time, increased deposition of liver glycogen and elevation of the blood sugar, as well as some degree of maintenance of the muscle glycogen may be achieved in the hypophysectomized animal, but this process is accompanied by large losses of nitrogen, so that increased gluconeogenesis would appear to be the source of the carbohydrate (Long, Katzin and Fry, 1940). Further, Bennett and Perkins (1945) have shown that the adrenal cortex is not necessary for the maintenance of muscle glycogen by pituitary extracts. Maintenance of the glycogen levels of the muscles during fasting appears then to be a function of the growth hormone unaccompanied by adrenocortical factors.

In attempts to demonstrate the activity of pituitary preparations on glycogen in normal fasting animals, significant effects have been seen in some instances, particularly in the heart (Illingworth and Russell, 1951). However, in other tissues the results on the whole have not been very impressive. This may be, of course, because the normal animal is already supplied with adequate quantities of hormone for its fasting metabolism. It is tempting to speculate that during fasting, when there is need for conservation of both carbohydrate and protein, there is increased secretion of growth hormone by the pituitary gland. The increase in cardiac glycogen which occurs normally during fasting but which is lacking after hypophysectomy might be considered particularly to support this suggestion, for otherwise it is hard to find an explanation of this phenomenon.

So far we have considered the effects of pituitary hormones on the maintenance of preformed glycogen in the tissues rather than its deposition from exogenous sources. When

carbohydrate is fed to hypophysectomized animals, it disappears from the body at something more than twice the rate seen in normal animals, despite the low metabolic rates of these animals (Russell, 1938c). Again the R Q. is high. It is not known at present whether the rapid disappearance of carbohydrate is due to increased oxidation or to fat formation in excess of its utilization, but there is some evidence to indicate that the latter may be important. The administration of pituitary extract or of purified growth hormone to normal animals before the feeding results in increased deposition of glycogen and lowering of the R.Q (Russell, 1938a; Milman and Russell, 1950; Illingworth and Russell, 1951). As shown in Fig. 1, the effect on the glycogen of the heart is particularly striking, for normally on glucose feeding there is little further deposition of glycogen in this organ.

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from the medium or in some cases its disposition by glycogen formation. In this respect the conditions are analogous to those employed in the intact animal fed carbohydrate. In general, the diaphragms from hypophysectomized animals have been reported to take up more glucose and to form more glycogen than those from normal animals, while the tissues from rats given pituitary preparations 18 or more hours previously *have exhibited diminished carbohydrate uptake and*

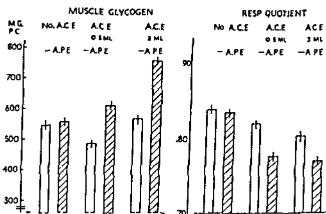


FIG. 3. *Effects of* anterior pituitary extract and adrenocortical

storage. These results are difficult to reconcile entirely with those made in intact animals; in fact, in respect to glycogen formation they are quite the opposite of those described above.

There are several possible explanations for this paradox. Krahl has suggested that there are two or more different pituitary factors concerned one which is slow to act (or is slowly converted in the body to an active form), which inhibits glucose uptake, and one or more which act more quickly to increase glucose uptake but to depress its utilization Presumably *the latter effects in combination could account for the*

ıcreased glycogen deposition when this is seen. Judgment s to whether this view is tenable depends upon a satisfactory eparation of the several factors.

In this work, it has been assumed that the uptake and phoshorylation of glucose by the tissues is the critical reaction, nd, other things being equal, that glycogen formation would e proportional to this rate Actually, available observations n glycogen formation as compared to glucose uptake by tissues f hypophysectomized animals or of treated animals are oo few to allow any accurate conclusions to be drawn. Recant 1952) has reported briefly that growth preparations added *n vitro* will depress the R.Q. but not the uptake of glucose rom the medium. If this is true, it would seem likely that here should be also some accumulation of carbohydrate in he tissues over that seen in the untreated slices. It is still ossible that under suitable conditions the effects of growth ormone *in vivo* on the disposition of carbohydrate may be luplicated in the isolated tissue.

In further work on this problem, attempts have been made o demonstrate an action of the growth hormone *in vitro*, not n glycogen formation but rather on the maintenance of preformed glycogen. In this work, the animals were first fasted and then fed glucose, in order to raise the initial level of glycogen in the tissue. Krebs' phosphate buffer was the medium employed. As shown in Table I, during incubation of the isolated diaphragm in the presence of growth hormone, there

Table I

EFFECT OF GROWTH HORMONE *in vitro* ON THE GLYCOGEN OF RAT DIAPHRAGM

	Glycogen—mg per cent*	
	Found	*Change during 90′ incubation*
Initial (15 min equilibration)	945	
Final, plain	598	−357
Final, G H (100 μg/ml)	719	−226
G H. effect		+121 ± 16

* Average of 12 experiments The average standard deviation of 16 duplicate observations under these conditions was 33 mg per cent

was in fact a significant conservation of glycogen. This seemed a confirmation of the *in vivo* experiments, but the problem has since developed some puzzling aspects. By the use of the anthrone reaction, it is possible to determine conveniently not only glycogen but also the total carbohydrate of the system. With this method, it was found that during incubation of the diaphragm there was not only disappearance of some carbohydrate, as was expected, but that there had been also a considerable conversion of glycogen to other forms of carbohydrate (Table II). It has not yet been possible to identify this intermediate, but it seems likely that it would be one or more of the phosphorylated sugars.

Table II

CHANGES IN TOTAL CARBOHYDRATE AND GLYCOGEN DURING INCUBATION OF RAT DIAPHRAGM *in vitro*

	Total carbohydrate (10 exp.) *mg per cent*	*Glycogen* (8 exp.) *mg per cent*	*Difference* (8 exp.) *mg per cent*
Initial (15 min equilibration)	968	950	20±25
Final (90 min further incubation)	825	525	323±18
Change during incubation	−143 (±26)	−425 (±41)	+302±37

When growth hormone was added to the medium and measurements made of the final concentrations of glycogen, total carbohydrate and lactate, the glycogen effect was still seen as before, but no changes occurred in the total carbohydrate or lactate balances (Table III). Hence, there appears to have been inhibition by the hormone of the conversion of glycogen to other forms of carbohydrate. Attempts to determine the effects of the hormone on the amounts of organic phosphate present have been frustrated so far by the fact that in bicarbonate buffer with some phosphate present the growth hormone effect is irregular and that in very low phosphate media it fails entirely. This point is, of course, to be investigated further. It is too early to say, if this inhibition of

Table III

GROWTH HORMONE EFFECT *in vitro* AND TOTAL CARBOHYDRATE

	Change in final concentrations (mg per cent) of			
	Glycogen	*Total carbohydrate*	*Difference T C —Glyc*	*Lactate*
Growth hormone (100 μg /ml) (7 exp.)	+118 (±19)*	−45 (±38)	−162 (±43)	−6 (±12)
Beef serum albumin (6 exp.)	−29 (±40)	0 (±45)	+28 (±28)	

* Standard error

glycogenolysis by the hormone is real, what may be its physiological significance.

In summary, then, it appears that we are still far from understanding the mechanisms of hormonal control of carbohydrate storage. We have a fair picture of events in the intact animal but even here our information is still quite incomplete. Work with surviving tissues has in some respects confirmed the *in vivo* observations, but in other areas there are unexplained discrepancies between the results obtained by the two methods of study. These suggest that more careful consideration of conditions in the intact organism and more detailed examination of the isolated tissue systems are both required

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DISCUSSION

HAGEDORN: In most text-books you will find remarks concerning the mobilization of glucose from the effects of adrenaline. It is explained that glucose is liberated in the liver and goes to the periphery. In order to see how much, I made these experiments.

Fig. 1. I injected 0 5 mg of adrenaline into a normal healthy man, fasting, and measured the arterio-venous difference between capillary

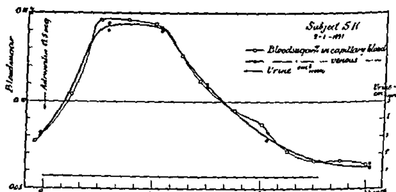


FIG. 1 (*Hagedorn*). Blood-sugar curves (capillary and venous) in normal fasting man into whom 0 5 mg of adrenaline has been injected

blood and blood from the median vein You will see that nothing is taken up in the periphery, so the explanation that glucose goes to the periphery won't hold true. However, there is the possibility that it

Fig. 3. You see there is a rise from ingested glucose, then the adrenaline is injected and it seems then that some glucose is liberated *from the periphery.*

I think this experiment agrees very well with what we heard the other day about the uptake of glucose and the effects of adrenaline with liver slices. It is seen to be only a change of equilibrium.

RUSSELL: I don't know, but if glycogen tends to go up, it is not a static effect.

BEST: You led us into this, I think, and we look to you for guidance

RUSSELL: It may still be a glycostatic effect, though. Normally there may be a balance between formation and breakdown of glycogen;

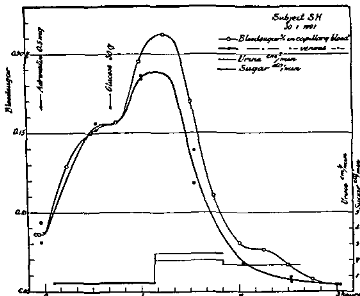


FIG. 2 (*Hagedorn*) Blood-sugar curves (capillary and venous) in same subject as Fig 1, with glucose administered three to four hours after adrenaline injection

then a glycostatic effect, i e an inhibition of utilization, with normal formation could result in accumulation of glycogen Even if the

ere has been

was neces-

sary in the

fasting animal

BEST. I warned Dr. Wilhelmi that I was going to ask you if you could get this effect without any insulin present in the body

RUSSELL: It hasn't been tried, as far as I know

BEST I was interested, particularly after your work, to discuss heart glycogen, because as you know this does go up in the absence of insulin.

RUSSELL: Yes, it does, and I am wondering if that might be evidence of increased secretion of pituitary growth hormone in the diabetic animal

LAWRENCE: I'm interested in the effect of thyroid on the glycogen of the heart, which McCance and I showed years ago to be greatly reduced. I'm wondering if any more has been done on that?

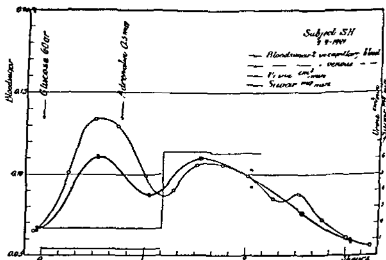


FIG 3 (*Hagedorn*). Blood-sugar curves (capillary and venous) in same subject as Fig. 1 with glucose administered first and adrenaline injected three to four hours later

one on the

heart and
muscle glycogen go to very low levels.

LAWRENCE. Well, at similar times, the skeletal muscle was little affected, and the heart rapidly depleted The liver is also very low in

fortnight or 20 days, there is no glycogen in the heart at all. In between, if you kill animals every 2 days you can see the glycogen-content of the heart getting less You find every figure between the normal and that in the heart at death from hyperthyroidism We saw the heart muscle, in some patients, it had an entirely different colour The heart muscle of a patient dying from thyroid crisis after operation is of quite another colour from the normal heart

RUSSELL: Some years ago, we found that the glycogen in liver and muscle of fasting thyroidectomized animals was perfectly normal, and that we could obtain increased deposition of muscle glycogen with [illegible] owth

[illegible] yco-
n to
be glycogen I had a similar experience with other tissues. Apart from glycogen, tissues contain some anthrone-reactive material which be- [illegible]

to identify this material I have tried to find if growth hormone affected the organic phosphate in this fraction, but as this effect of the growth hormone is best seen in a phosphate medium and it is [illegible]

that practically all the carbohydrate is glycogen initially It is in the incubated tissue that you see this peculiar change

LUKENS What other tissues than the brain, and what other animals or plants use carbohydrate without forming glycogen?

BEST Reptiles

G. CORI The brain has some glycogen.

LUKENS Yes, but that's in very small amounts

G. CORI It disappears very rapidly, but you can determine that it is actually there

LUKENS: You mean it disappears more rapidly than glycogen in other places?

G CORI Starting at higher original levels, it disappears in other tissues rapidly too

RUSSELL Particularly in the heart It disappears there within seconds of opening the chest cavity

LONG It is interesting that the atypical behaviour of the muscle glycogen is apparently present in two tissues that are in a state of continuous activity, that is the diaphragm and the heart

RUSSELL. They do it rather differently

LONG Yes, but your effects are very marked in the contractile tissues that are in continuous activity.

RUSSELL But they have a higher metabolic rate probably than the gastrocnemius would have at rest.

[illegible] of cortical extracts [illegible]

pituitary fraction which you can't see otherwise.

MANN: With refe[illegible] other tissues than brain can use [illegible] mention spermatozo[illegible] glycogen (either in [illegible] seminal fructose at a very high rate, converting it to lactic acid. It is interesting to note the clear division in function in so far as carbohydrate metabolism of semen is concerned the sperm cells provide the enzymic equipment but have no carbohydrate The carbohydrate is provided entirely by the cell-free surrounding medium (the seminal plasma) in the form of fructose

CONN· This is comparable to the diabetic man who increases his heart muscle glycogen and also increases his renal tubular glycogen Have there been any studies with respect to growth hormone on renal glycogen?

RUSSELL No, we haven't done any and I don't know of any

BEST He may also increase his "pancreatic" glycogen.

RUSSELL I think that perhaps we should have looked at some more organs, a number of analogies would have been useful

BEST. The change wouldn't have been very great.

MIRSKY The kidney glycogen change could be attributable to [illegible]

effects are due to increased insulin secretion. One can get the effects of the growth hormone with quite small amounts which do not have [illegible]

parallel.

BEST· No It would be useful to do all these things over again on diabetic animals.

RUSSELL Yes, but I just haven't got around to that.

PART V

THE INFLUENCE OF INSULIN ON CARBOHYDRATE METABOLISM

THE INFLUENCE OF INSULIN ON CARBOHYDRATE METABOLISM

C H BEST

This is a subject to which I have given considerable thought over some thirty-one years, but I shall delay putting my present ones in some sort of order until after I have read the excellent papers on closely related subjects which will form the basis of our continued discussions this summer. Many aspects of the subject assigned to me can be described by the investigators who actually made the advances, and I shall use my position as Chairman to attain this desirable goal. There are many stimulating recent developments which are clear cut under the conditions used for their demonstration. The part which each of these may play under more physiological conditions will of course provide interesting opportunities for further work

It was natural that Banting and I should ask ourselves, in [illegible] cause of the decreased sugar excretion?" We knew that the liver failed to store glycogen, and that the respiratory quotient failed to rise when sugar was given to a depancreatized dog. Actually our first thought on the action of insulin was that it caused more sugar to be burned What a hectic course this

idea has followed over the years! With our colleagues, particularly Dr. John Hepburn, we did record the rise in the respiratory quotient of the diabetic dog and man following insulin administration. We were, of course, ignorant of the very rapid conversion to fat, which is greatly accelerated by insulin. Dr. Lukens has reviewed this subject in some detail, and it may well have accounted for a significant part of the rise in R.Q. which we noted. Many of you will be familiar with Professor J. J. R. Macleod's views on this subject as expressed in his books "Insulin and Carbohydrate Metabolism" and "The Fuel of Life." He supported overproduction rather than under-utilization as the mechanism of production of diabetes Dr Samuel Soskin has been perhaps the most ardent advocate of these views among Professor Macleod's pupils.

I have consistently taught that insulin increases the oxidation of sugar, but I admit that evidence to satisfy our present standards has only recently been secured. It is, of course, not certain that our present evidence will satisfy our future standards Villee and Hastings (1949) found that insulin increased the oxidation of glucose as judged by the appearance of ^{14}C from labelled glucose in the carbon dioxide formed by the isolated rat diaphragm Similar results have quite recently been reported by Sacks and Sinex (1952), who found that insulin increased the turnover of ^{32}P in A.T.P., phosphocreatine and hexosemonophosphate, the deposition of ^{14}C glucose as glycogen, and the oxidation of the labelled glucose, in the isolated rat diaphragm.

The findings on isolated tissues have been extended in an interesting and convincing manner by Wick, Drury, Bancroft and MacKay (1951), who by infusing ^{14}C labelled glucose into eviscerated rabbits were able to distinguish between the glucose which disappears and that which is oxidized One surprising finding was the small proportion of the glucose which disappears that is oxidized or stored as fat or glycogen. These routes of disappearance account for less than one quarter of the total. When insulin was given there was a

arge increase in the ^{14}C of the expired air. The extra glucose disappearing under the action of insulin when it was not oxidized, was disposed of as non-glucose water soluble compounds, with smaller amounts found in glycogen, protein and fatty acids.

Dr. Lukens was actually named in our programme to speak on the effect of hormones on fat synthesis from carbohydrate and I have, therefore, no responsibility to discuss the action of insulin in increasing the disappearance of glucose by this route. I suppose I do have some responsibility to discuss the effect of insulin on oxidation of glucose, but I will invite Dr. Drury to supplement the remarks which I have made, and to answer questions that may be asked on his work with Doctors Wick, Bancroft and MacKay These workers have undoubtedly attained the most advanced position with respect to our knowledge of the fate of glucose under the action of insulin.

It was easy to show in the diabetic dog that insulin caused great increases in liver glycogen. I remember a value of over 20 per cent in one of our first dogs which received large amounts of both glucose and insulin. The demonstration of an increase in muscle glycogen was, as Dr. and Mrs. Cori will remember, more delayed Muscular activity induced by the hypoglycæmia, was a complicating factor, and until this was overcome by the appropriate administration of sugar or by the use of eviscerated or denervated preparations, a convincing increase of glycogen in muscle tissue was not secured. I have not looked up this ancient literature, but as I remember it, the Coris were using one type of preparation and the group working in H. H Dale's laboratory—which included J. H. Burn, and a little later J P. Hoet and myself—were using another. Gemmill's work on isolated rat diaphragm did not appear until 1940, and you all know what a great advance this preparation has made possible. However, it had, of course, been well established before this that insulin did promote the synthesis of muscle glycogen. Dr Jane Russell has now brought us up to date on the hormonal control of

glycogen formation and has thus covered one aspect of the action of insulin on carbohydrate metabolism.

I will not attempt to review the reactions through which glucose passes in muscle, in the presence of such great contributors to this field. I hope that Dr. Cori, Dr. Krebs, Dr. Dickens, Dr de Duve and others will participate extensively in this discussion of how insulin acts. The glucokinase reaction—the first of the series—is essentially irreversible, and is considered by many authorities to be the limiting factor in glucose uptake in muscle extracts. The glucose that disappears from the medium surrounding the isolated diaphragm goes to form glycogen, lactic acid or CO_2, and until some effect on subsequent reactions can be specifically demonstrated it is assumed that factors which affect the rate of appearance of any of these products are acting on the phosphorylation of glucose. Gemmill (1941) showed that a little more than half the glucose disappearing in the normal rat diaphragm appeared as glycogen; Walaas and Walaas (1950) found about one quarter as lactic acid; and Bartlett, Wick and MacKay (1949) and Villee and Hastings (1949) secured the equivalent of up to 10 per cent as CO_2. Insulin stimulates the uptake of labelled glucose by the diaphragmatic muscle, the deposition of glycogen, and the formation of CO_2. Insulin does not cause a significant glycogen formation from pyruvate. The results obtained with liver slices parallel in general those obtained with muscle.

Dr. Folley has reviewed the effect of insulin on glucose uptake and on lipogenesis *in vitro* in mammary gland tissue In adipose tissue, *in vivo*, insulin promotes the formation of glycogen (Wertheimer and Shapiro, 1948) and fat (Chernick and Chaikoff, 1951; Wick, Drury, Bancroft and MacKay, 1951).

Thus we have good evidence, some of it dating back many years, that insulin stimulates the glucose uptake of muscle, liver and adipose tissue. The suggestion was made many years ago that insulin might lower the permeability of the cells to glucose, and this change might completely or in part account

for the increased utilization of glucose. This matter has been explored recently by Levine and his collaborators (1949), and by Wick. The former group showed that in the eviscerated, nephrectomized animal, in the absence of insulin galactose is distributed in a system equivalent to 50 per cent of the body weight With insulin the distribution was throughout a volume corresponding to all the water of the body. It was postulated from these data that the action of insulin in facilitating sugar entry into muscle was exerted on the cell membrane and not upon the intracellular enzyme systems Wick found no effect of insulin on d-sorbitol distribution. Goldstein, Henry, Huddlestun and Levine (1952) have strengthened their position considerably by the finding that sugars which possess *the chemical configuration exhibited* by glucose in its first three carbon atoms have their distribution increased by insulin, i e., presumably they pass into the cells. Enzymic mechanisms may of course, be responsible for the increased sugar entry, and these could operate from one or more of the three possible sites. The recent interesting paper of E J. Ross (1952) on the accelerating effect of insulin on the permeability of the blood-aqueous barrier, can now be considered with the finding of Levine and of Wick The presence of a special enzymic mechanism for the activated diffusion of glucose, situated on the membrane of the cells, is supported. Professor Frank Young will be particularly interested in and be able to discuss this work

Now the only phase of insulin action on carbohydrate which I was quite sure would not be covered before my turn came, was the inhibition of new formation in the liver This is still in an unsettled state when judged by the results obtained in the experimental animals, but Dr. Bondy and Dr. Sheila Sherlock have obtained more convincing findings in diabetic human cases, and I shall leave this field to Dr. Sherlock who will perhaps give her interpretation of the experimental findings in animals as well as in man

The combination of insulin with muscle, adipose, and mammary tissue, so fruitfully explored by Stadie and his group

(1951) will perhaps be one of the features on which our attention will be focused.* Also, the inhibition of many of the effects of insulin by unknown and known pituitary and adrenal factors, and by combination of these, in which scores of people are interested, provides us with fine ammunition. This subject has been exceptionally well reviewed by Krahl (1951).

These pituitary and adrenal factors which appear from many of our experimental approaches to be antagonistic to insulin, normally of course exert synergistic and controlling influences for the maintenance of the physiological state. It is of interest, however, for the experimentalist to concentrate on one factor in the absence of others. Mr. James Salter and I have been concerned recently with the effects of gradually increasing doses of protamine zinc insulin in hypophysectomized animals in which the adrenal cortices and thyroid have at least partially atrophied We have induced growth of as much as eleven grams per day in animals which had been "plateaued" at 90 grams for several months on a complete diet available *ad libitum.* None of the other *growth* hormones that have been studied operate anabolically in the absence of insulin. Insulin may induce growth—quite probably not maximal balanced physiological growth, but retention of nitrogen—deposition of glycogen and fat, and widening of the epiphysial line of the tibia, in these hypophysectomized preparations Removal of the anterior pituitary alone results usually in *zero* growth. Removal of all the insulin-producing tissue results in, shall we say, "negative" growth. We will report the effect of insulin on the growth of Houssay animals later. It will probably be similar to that of hypophysectomized animals.

One of the most interesting possibilities which this work emphasizes is that *one* of the effects of removal of the pituitary is to decrease the rate of liberation of insulin The established loss of "growth hormone" and the relative increase in insulin

*Bleehen and Fisher (*Proc. physiol Soc*, July 1952) have recently suggested, on the basis of findings in perfusion experiments, that this combination of "externally applied" insulin with muscle is unphysiological.

effect may have obscured an absolute loss of insulin. Many of these possibilities are being investigated I am hoping that these preliminary findings will stimulate discussion and perhaps some re-orientation of thought on which endocrine product is the most fundamental or indispensable growth hormone. I would emphasize again that physiological balance has been disturbed under many of our experimental conditions, and that our efforts must be continued in the hope of learning more about the action of the various hormonal growth factors under physiological conditions.

The results already emphasize the point that an anatomically intact pancreas does not always supply sufficient insulin to produce the maximum effect on growth which this hormone is capable of exerting. The clinician will undoubtedly cautiously explore the possibility that these findings be applicable in the treatment of various types of dwarfism.

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DISCUSSION

WILHELMI: Have you studied the composition of the weight gain in your insulin-treated hypophysectomized animals?

BEST: Yes, but it's only been studied by chemical analysis of the 10 rats on each side. In completely homogenized tissue, on the average in

(1951) will perhaps be one of the features on which our attention will be focused.* Also, the inhibition of many of the effects of insulin by unknown and known pituitary and adrenal factors, and by combination of these, in which scores of people are interested, provides us with fine ammunition This subject has been exceptionally well reviewed by Krahl (1951).

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Dr. R H. Smith certainly got some growth under those conditions, but whether we would have got the same with insulin alone we have not tried because we rather feared the effects of hypoglycæmia on the animals Would you think it possible that glucagon might be a signifi-
e in danger

among your rats?

Best: Yes, I talked about this at the Lilly symposium, and Li went home and gave regular insulin to hypophysectomized rats and killed them all Then he wrote to me protesting that he couldn't repeat the experiments. I'd forgotten to tell him that Salter had had the same experience, that they had all died with regular insulin, and that with

the high rate of survival

Long: Dr Best, I believe that there might be another interpretation of your work. Samuels, you will recall, force-fed hypophysectomized rats up to the calorie level of the normal animal I remember he found, of course, a marked increase in body weight, and at the same time there was some retention of protein under those circumstances You can't be sure that the high level of intake did not increase the insulin secretion.

Best. Well, isn't that the answer—do it in the diabetic animal and see what you get I mean, force-feed a completely insulin-free animal and see how much weight-gain there is There won't be any at all

Long In the Samuels experiments, no growth hormone was given

Best No. That work was challenged by Levin, who got a different pattern—there was more increase in fat and less increase in protein

Russell Levin found no increase in protein, in fact there was a small loss There was a large increase of fat in the bodies of his force-fed hypophysectomized animals

Best I'm interested in this work particularly because it forces us to do so many different things That's why I asked Hugh Long if we could give insulin to adrenalectomized animals and how long we could keep them alive without any adrenal corticoids, and still have them eat well. What will the pattern of growth be there?

Long. Well, it would be easy there, because you could put in a pellet of DCA and then they'd sit up and you could give them lots of food.

Best: But would that be fair? We may have to try the experiment with a high salt diet and no cortical material

Conn In the hypopituitary dwarf that I described earlier, food intake was constant On insulin alone, there was nitrogen retention of 1½ g./day, with constant food intake.

Best: I think perhaps that the most interesting thing that comes out is that there is a possibility, if we get our blood assay going well, of finding the level of blood insulin in hypophysectomized animals I'd

12 to 15 days there was an increase of 4–7 g. of protein per rat and quite a large increase in fat and water. I have the figures available if you wish to look at them. Mr. Salter has a second study of this kind going on now.

LONG: May I ask if the food intake was increased by the administration of insulin?

BEST: Yes, as it is with the growth hormone. You could not give these large doses of insulin if the food intake were not increased.

LONG: You haven't done any experiments comparable to that of Li and Shaefer, where the food intake was restricted?

BEST: When you restrict the food intake with growth hormone you do get a definite growth but much less than with unrestricted food. You can al
with insuli
remember
doses of insulin

LONG: I remember that in the Li and Shaefer paper, they used groups of rats. On an equal calorie intake over a period, the weight gain in the animals treated with the growth preparation was approximately twice that of those who did not receive it.

WILHELMI: One point is that when animals are treated with growth hormone, the p[illegible] and that of
fat is diminishe
show that you

BEST: I thin
of fat and less of protein, but that there is a very definite increase in the protein.

WILHELMI: Well, I think that the patterns of growth, if we describe both of these processes as growth, are decidedly different.

BEST: Maybe, but we have to know how much extra insulin the growth hormone liberates. Perhaps that's a better way of liberating insulin, giving growth hormone, than giving it the way we did in one dose a day. We have to know more about this before we conclude that the growth hormone effect is a direct one, independent of the action of insulin. The two may prove to function independently but also synergistically. There is however little evidence of the growth effect of growth hormone in the absence of insulin.

MIRSKY: Is it not possible also that the growth hormone inhibits lipogenesis and thereby makes glucose available which in turn stimulates the pancreas, so that you have a different distribution when growth hormone is administered to the intact animal?

BEST: Yes, I think the pattern of growth may well prove to be different.

MIRSKY: But the insulin still remains a growth factor.

BEST: Well, it is a growth factor in the complete absence of the pituitary, obviously. Growth hormone can be described in various ways.

YOUNG: We did some experiments in my laboratory in the hope that we might get good growth with hypophysectomized rats treated with the *a* cell hyperglycæmic factor (glucagon), together with insulin

hyperglycæmic factor—glucagon. Do you accept the evidence which Bornstein, Reid and myself got, that growth hormone may elicit a secretion of glucagon from the pancreas?

BEST: I didn't find it very convincing

YOUNG Would you think that in your experiments, the influence of insulin is sufficient to account for the whole effect of growth hormone on growth, provided you could give it under physiologically satisfactory conditions?

BEST: I do not think so We are drawing *no* physiological conclusions from this type of experiment Physiologically satisfactory conditions might demand the presence of all the other hormones

YOUNG. It is conceivable, only there is something else which might be antagonizing, in part, the hypoglycæmic action of insulin under these conditions.

BEST: One could, of course, make a good story on the evidence available, of their being three or more hormones necessary to antagonize insulin to keep things under control There was hyperglycæmic factor in the insulin we first used, and your question is a very good one. I don't know how far you thought your own results were convincing in this respect *I think you suggested that there was liberation of* hyperglycæmic factor but I don't think you said that this was your definite conclusion.

YOUNG We did not conclude as certain that the hyperglycæmic substance did indeed come from the α cells of the pancreatic islets, but it was clear that blood from the pancreatico-duodenal vein gave a hyperglycæmic effect under these conditions

BEST: It's so easy to get non-specific effects, don't you think? I didn't feel very convinced that the findings were really due to the

adrenalectomized (ADHA) rats to which 1 ml of blood from cats with growth hormone-induced diabetes was administered intravenously The curve labelled P-vein is that for the blood-sugar changes in the

paper.

effect.

MIRSKY. A number of years ago we demonstrated that the removal of amino-acids from the circulation was increased by insulin, while the

like to know this very much because the pancreas looks normal, as

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LUKENS: What was the age of the rats which you used?

BEST: They were from 90–110 grams.

LUKENS They must have been young then, about how many days?

BEST. I can't remember exactly. As a matter of fact, the first ones we grew ourselves, later we bought them from the hormone assay people.

LUKENS: So they'd be about 40 or 50 days? Rats grow after hypophysectomy up to about the age of 40 days.

BEST We had controls on all of them One group analysed at the start of the experiment and the two groups, one with insulin and the other without, both on the same diet—as much as they would eat—analysed at the end of our experiment.

LONG: I believe you and your colleagues showed that the pancreas of the hypophysectomized animal also responds to dietary changes, as the normal animal does? This would imply that there is an independence of insulin secretion from pituitary control.

BEST: Yes

YOUNG: Did you have to give glucose to your hypophysectomized insulin-treated rats?

BEST. They had to have what they considered an attractive, high

point.

SMITH. Is there any effect of insulin or growth hormone on nucleic acid synthesis in tissues which are depositing protein under the influence of these hormones? For nucleic acid synthesis, obviously you are going to need pentose or pentose-phosphate Professor Dickens, in the first paper at this meeting, devoted much of his time to discussing the breakdown of glucose to pentose and I was wondering whether there was any effect on this reaction It is one we have not discussed much so far and it may perhaps be relevant to this question of nucleic acid synthesis

DICKENS There seems to be a great deal of doubt about whether growth hormone has anything to do with increased nucleic acid metabolism. From the name alone it really ought to have something to do with this, but nobody seemed to know anything about it in our earlier discussion on this point

YOUNG: I would like to come back again to the question of the

glucose and got a bit of glycogen in the liver. He concluded that it was formed without insulin.

RUSSELL: You can get glycogen in the liver of the depancreatized cat without any insulin for several days.

LAWRENCE: It took Groen in Holland, with depancreatized dogs, 21 days before he could demonstrate no insulin, according to the rat diaphragm mechanism

[illegible]

vations about 2 hours after operation, when the major part of the insulin is gone and the blood amino nitrogen is in the stage of steady increase If amino-acids are given to such an animal treated with

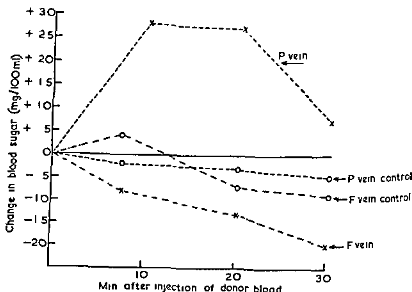


FIG 1 (*Young*) Influence of blood from growth hormone-induced diabetic cats on the blood sugar of recipient ADHA rats.

growth hormone, the level to which the amino nitrogen falls is considerably lower in the treated animal than in the untreated animal. An effect of growth hormone on uptake of administered amino-acids is obtainable in the absence of extra insulin

BEST: Yes, but actually, with the level high enough you might get deposition without either growth hormone or insulin. I was very impressed by Krahl's findings, he reported how very careful you had to be to get rid of the insulin Three or four days was necessary without insulin to get rid of the last trace, as tested by glucose uptake of the diaphragm. I remember a paper of Dr. Mann's with depancreatized dogs. He left them without insulin for 24 hours, then gave large amounts of

In this preparation we have found that the combustion of glucose, i.e., its complete oxidation to water and carbon dioxide, is increased by insulin, but the effect develops relatively slowly. This action can be shown by giving the animal labelled glucose and comparing the radioactivity of the respired CO_2 with that of the circulating sugar. To carry out this test it is necessary to keep the activity per mg. of blood sugar constant throughout. To get such a constant activity, we give first a priming dose of rather potent [^{14}C] glucose, which after mixing will give a certain activity to the circulating glucose. A mixture of labelled and of ordinary glucose is then made up to have this same activity per mg., and a solution of this is given continuously in such amounts as to maintain a normal blood sugar level. The exhaled CO_2 is collected and the activity of it is determined and compared with the activity of the blood sugar. In this way we can determine the amount of glucose oxidized. The activity of the exhaled CO_2 is not at first the same as that of the CO_2 given off by the tissues. The latter is diluted by the body CO_2 at first. It takes about one and a half hours for equilibrium to be established between these two. In other words, if the tissues start producing CO_2 of a certain activity and continue to produce it with this same activity, after one and a half hours the activity of the exhaled CO_2 will be practically the same as that being produced by the tissues.

Fig. 1 shows the results in 2 rabbits studied in this way. One rabbit was given no insulin, and the other was given insulin in supramaximal doses throughout. We see that in the latter there was a definite increase over the control in the amount of glucose oxidized. The amount of this increase is not maximal at first but needs at least 8 hours to develop fully. On the other hand, the glucose disappearance rate in the insulinized animal is markedly increased over the control from the first, and the rate at which glucose must be supplied to these two animals remains almost the same throughout the 8 hours.

We can, therefore, increase the rate of oxidation of glucose

CAN OTHER FUELS SUBSTITUTE FOR GLUCOSE IN TISSUES SUBJECTED TO INTENSE INSULIN ACTIVITY?

*D. R. DRURY and A. N. WICK**

IN their original work Banting and Best showed that insulin promotes the removal of glucose from the blood into the cells. This raised the question—does insulin increase the immediate and direct combustion of glucose by the tissues? Subsequent work in several laboratories showed that insulin causes a rise in the respiratory quotient. Such a rise may result from an increase in direct oxidation of glucose, it can also be due to an increase in the conversion of carbohydrate to fat. There is even the possibility of the transformation of carbohydrate to fat taking place in a special organ like the liver, and the burning of fat simultaneously by the general tissues of the body; such a combination could give an R.Q of 1.

By the use of glucose labelled with radioactive ^{14}C it should be possible to tell definitely whether insulin increases the oxidation of glucose by the extrahepatic tissues. We have been studying this in the eviscerated rabbit. We have usually also removed the kidneys since these organs seem capable of carrying out at least some of the chemical reactions usually considered only to take place in the liver. In such a preparation insulin produces the effect shown by intact animals: it promotes the transfer of glucose from the extracellular space into the cells. This effect is immediate, i.e., the disappearance of glucose from the blood is accelerated right after the injection of insulin. This can be measured by determining the rate at which glucose must be supplied to the animal in order to keep the blood sugar constant.

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by giving insulin and glucose. There are certain foodstuffs such as β-hydroxybutyric acid whose oxidation by the animal can be caused simply by increasing their concentration in the blood. We have been studying the relative rates of oxidation of glucose and of β-hydroxybutyrate when they are given together to animals under maximal insulin activity.

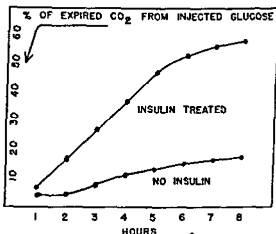


FIG 1. Graph comparing the amount of CO_2 coming from glucose in the insulinized, and in the non-insulinized, animal.

Fig. 2 shows the results of such an experiment. For the first 5 hours the animal received maximal doses of insulin, and glucose in amounts just adequate to keep the blood sugar at a constant normal level. When β-hydroxybutyrate is added there is an immediate decrease in oxidation of glucose. The rate of disappearance of glucose from the blood is not changed by the addition of this extra fuel.

Acetate gives similar results and a typical example is shown in Fig. 3.

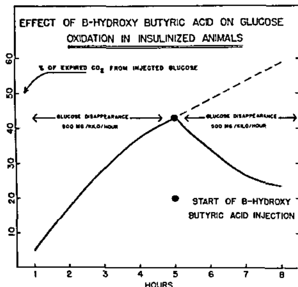


FIG. 2. Graph showing effect of addition of β-hydroxybutyric acid on glucose oxidation in insulinized animal Injection of β-hydroxybutyrate started at end of fifth hour. Solid line portrays actual results Broken line indicates expected results if β-hydroxybutyrate had not been superimposed.

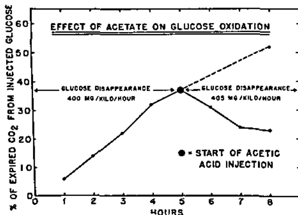


FIG 3 ... effect of add ... on of acetate ... sulinized ... shows a

It appears therefore that the extra-hepatic tissues under the influence of insulin will use certain fuels when offered them, and these will displace glucose as a source of energy. The rate of transfer of glucose into the cells however is not changed.

We are also studying the effect of insulin on the rate of combustion of these "competitive" fuels. In this case we must have this fuel tagged. We have been studying acetate metabolism in this way. A priming dose of 200–400 mg. per kg. of the labelled acetate is given at the onset. This is given as the sodium salt. Then a steady, continuous, injection of the acid is given at an hourly rate which equals 40 per cent of the acetate given in the priming dose. When treated in this way, the animals quickly attain a steady state with respect to elimination of tagged CO_2. Table I shows the results of a

Table I

Results Showing Rate of Oxidation of Acetate in Eviscerated Rabbit

Hour	*Specific Activity of Expired* CO_2	CO_2 *Expired mg /kilo/hr*	*Acetate Oxidized mg /kilo/hr*
1	91	805	89
2	186	725	164
3	177	795	170
4	164	803	160

Priming dose of 490 mg per kilo of labelled acetate given at start of experiment *Thereafter* 240 mg acetate per kilo given each hour by constant injection Specific activity of injected acetate = 715

typical experiment. The radioactive CO_2 is given off in the first 2 hours at rates as high as would be given were radioactive bicarbonate injected. This shows that the rate of oxidation of acetate develops very rapidly and is in marked contrast to the manner in which the rate of glucose develops

After we found that acetate is oxidized at a steady rate under these circumstances, we tried the effect of superimposing insulin midway through such a course Glucose was given throughout in amounts needed to keep the blood sugar at a normal level. We have carried this out in 8 animals. The

average for these are given in Table II. The 3 hours before and the 3 hours after insulin are shown. Insulin does not affect the rate of oxidation of acetate. Any fluctuations in activity of respired CO_2 are due to proportional changes in the total CO_2 exhaled. In these animals the requirement for glucose is increased in a normal fashion after the insulin had been given. Thus increased amounts of glucose went into the cells but none of this displaced acetate in combustion. Evidently the

Table II

AVERAGE VALUES FOR EFFECT OF INSULIN ON ACETATE OXIDATION*

	Hours	*Per cent of Administered Acetate Oxidized*	*Total CO_2 mg /kilo/hr*
Control Period	1	60	784
	2	60	776
	3	56	691
Insulin Period	1	63	777
	2	61	751
	3	52	660

*The hourly rate of acetate oxidation is expressed in percentages of the acetate injected each hour

cells burn acetate in preference to glucose even under maximal insulin activity. From the lack of any change we can infer that it is not a matter of competition but rather of displacement.

Insulin, therefore, forces glucose into the cell, which may promote its oxidation. However, such glucose does not inevitably proceed to immediate oxidation This follows from the fact that such glucose can be so easily diverted away from oxidation by giving these other fuels In other words insulin is not an oxidative catalyst. Actually, as far as the extrahepatic tissues are concerned, acceleration of transfer of glucose into the cell could be the sole action of insulin. This brings us more or less back to where we were when Banting and Best originally showed that insulin caused the removal of glucose from the blood. At any rate there is the view that

is frequently expressed nowadays which assumes that insulin merely accelerates the transfer of glucose into the cell. Once it has arrived there it is taken care of by the machinery of the cell and the route of its disposal is a local matter depending on the state of the cell. Our findings are consistent with this view.

Summary

In the eviscerated rabbit insulin increases the oxidation of glucose, but this increase is slow in developing.

Once an increased rate of glucose oxidation is thus attained it can be quickly and markedly depressed by the giving of β-hydroxybutyrate or of acetate. The rate of transfer of *glucose into the cell is not affected by giving the other* fuels.

Insulin does not affect the oxidation rate of acetate.

DISCUSSION

Mirsky: In order to test the mechanism of insulin action on glucose, we tested the effect of insulin on the uptake of sugars by the isolated rat diaphragm. Since only small quantities of some sugars are removed from the incubation media by the diaphragm, we used two hemidiaphragms for each test. When a single incubation period was used *we had much difficulty in determining whether insulin had an effect* or not. This was due to the fact that with some sugars the spread was great. Consequently, we studied the removal of the sugars by two hemidiaphragms incubated for different intervals up to four hours. Our results with glucose, galactose and fructose were as follows:—

Insulin produced a marked acceleration in the removal of glucose from the medium by the diaphragm. At 30 minutes the effect of insulin was just statistically significant and at 60, 120 and 240 minutes, the effect was highly significant. We were surprised to find that at incu-

MIRSKY: No; what disturbs me is that we get it with fructose.

G. CORI: I would like to ask Dr. Drury whether he has considered the effect of release of sodium ions when he injected the organic acids, and whether the flooding of the organism with sodium could not by itself depress sugar utilization; have you done controls with bicarbonate to exclude this possibility?

DRURY: We gave neutral salts to begin with and then we gave the acid as such at a rate which replaced that which was metabolized.

G. CORI: You mean you gave the free acid?

DRURY: Yes, we immediately started free acid as soon as we had given the priming dose of the salt

G. CORI: Did you determine CO_2 combining power in the plasma? You had no kidney there, had you?

DRURY: We did try to control it by giving bicarbonate

G. CORI: Did the injection of sodium bicarbonate not interfere with sugar utilization?

[illegible] ulate

[illegible] you

DRURY. I don't think so.

[illegible]

glucose uptake Thus half of the glucose is not used for glycogen production. It might be oxidized.

DRURY. Do you mean that there is a specific increase in the glycogen [illegible]

[illegible]

Is that known?

VERZÁR: I think Krahl *et al* did some experiments about this

MIRSKY. In that connection, it is interesting that if one incubates a rat diaphragm with nembutal in a concentration which will produce [illegible]

He then sampled the CO_2 of the atmosphere at various intervals by a technique which only takes a very small amount of gas so that it would not appreciably affect the total amount of CO_2, and measured its specific activity. To our surprise, he found the same rate of increase in specific activity whether or not insulin was present. Insulin didn't seem to affect the rate of the appearance of the labelled acetate carbon in the atmospheric CO_2 in the Warburg flask. It rather looks as though there is a lag in the transfer of the respiratory CO_2 from the cell into the atmosphere. In control experiments with labelled bicarbonate and no tissue, Dr Glascock has found that it takes a long time to get randomization of the label, that is to attain the maximum specific activity in the gas phase. I wonder whether the same sort of phenomenon applies in living animals?

DRURY: Well, was it a long time?

FOLLEY. I think it was about 30 minutes. It was longer than that before you got anything?

DRURY: Yes, in our first hour [illegible] that rate, and that is to be expect[illegible] at a constant rate intravenousl[illegible] don't get the same specific activ[illegible] injected bicarbonate in the first hour In the second hour it reaches approximately the specific activity of what you are injecting

CONN. What happens to the glucose which disappears? The presumption is that it is converted into glycogen; were there any respiratory quotient changes under those circumstances?

DRURY: We didn't do oxygen concentration. What it needs is a carcass analysis but of course that needs a lot of activity. Respiratory work is relatively simple because you don't need very much activity, and you get so much of the CO_2 in such a pure form

RUSSELL It's so diluted in the carcass.

DRURY: Yes, it's so diluted and you are dealing with compounds that are awfully hard to separate.

INHIBITORY EFFECT OF PANCREAS EXTRACT AND H-G. FACTOR ON THE INSULIN GLUCOSE UPTAKE OF THE ISOLATED DIAPHRAGM

J. L. R.-CANDELA

It is not an easy task to speak of the action of the hyperglycæmic factor of pancreatic origin, when the results obtained by the various research workers who have devoted themselves to this interesting problem are still scarce and not definite. We are still lacking sufficient elements of judgment to decide whether the factor which is attributed to the secretion of the α cells plays an important part in human diabetes, and we do not know either which is the mechanism of its action.

No sooner was insulin discovered by Banting and Best than MacLeod in 1922 pointed out a hyperglycæmic effect of the preparations used until then. Afterwards Murlin, Burger, Bürger and Brandt, Zucker and Berg were able to detect analogous hyperglycæmic effects Results *in vitro* have been obtained by Kepinow, Nielssen, etc , Sutherland and Duve which prove the glycogenolytic effect of the pancreatic extracts, as also, with less intensity, of the gastric and intestinal mucosa extracts. Heard *et al.* have maintained that it is the question of a hormone which comes from the α cells. Benard thinks that the glycogenolytic and hyperglycæmic substance would be a co-enzyme which would activate the hepatic glycogenolysis Sutherland and Cori show that the hyperglycæmic factor acts on the phosphorylase system Cavallero shows that the hyperglycæmic factor proceeds from the islands and not from the acinose texture.

An indirect proof of the existence of a pancreatic hyperglycæmic factor has been obtained by Thorogood and Zimmermann (1945), Candela *et al.* (1947) by showing that a dog suffering from alloxan diabetes requires less insulin after the

removal of the pancreas. It is not apparent that these results can be attributed to the lack of external pancreatic secretion with resulting trouble in the absorption, as the ligature of the ducts (Candela) does not produce any change whatever in the requirements of insulin. The removal of the pancreas after the ligature (Candela *et al.*) results in a reduction in the requirement of insulin. Bornstein, Reid and Young have very recently proved that under the influence of growth hormones, the islets of Langerhans liberate the hyperglycæmic substance which they contain.

Experiments

We have studied *in vitro* the action of raw pancreatic extracts and of the hyperglycæmic factor.* The effects *in vivo* of pancreatic extracts on the rat diaphragm prove the inhibiting effect on the glucose-uptake

The experiments were done as follows:—

A. Influence of the pancreatic extracts in the action of insulin on the glucose-uptake.

B. Influence of the pancreatic extracts (alloxan diabetic dog) in the action of the insulin on the glucose-uptake.

C. Influence of the hyperglycæmic factor (Lilly) in the action of insulin on the glucose-uptake.

D. Insulin glucose-uptake from rat isolated diaphragm previously incubated in a medium which contains the hyperglycæmic factor.

Material and Methods

We have always followed the same technique, mentioned in a previous work. White rats from 100 to 150 *g.* weight are used, which after being beheaded have their diaphragm removed, and this diaphragm is divided into four parts, the *pars tendinosa* being eliminated as much as possible. After weighing it in torsion scales it is introduced into the glass which contains the buffer. In cases A and B the buffer was

*We thank Dr. W. R. Kirtley of Lilly Research Laboratories for the supply of hyperglycæmic factor and insulin

prepared in a concentration which when mixed in equal parts with the extract would give the appropriate concentration. The raw extract was prepared by taking the pancreas immediately after the dog's death and making a vacuum concentration in a temperature not over 45°C. One cc. is equivalent to 10 g. of raw pancreas.

In the case of section C we used the hyperglycæmic factor furnished by E. Lilly in a dose of 0·0001 g. for each diaphragm dissolved in the buffer. With section D, the same dose of factor was contained in 2 ml. of buffer without glucose. The diaphragm was incubated as was done by Stadie for two minutes at 25°C After washing, it was put into the buffer with glucose and insulin.

In cases A and C the order of the experiment was as follows:—

First Diaphragm + buffer + pancreas extract + insulin
Second Diaphragm + buffer + pancreas extract
Third Diaphragm + buffer + insulin
Fourth Diaphragm + buffer

In case B, the same as in the aforementioned cases, but the buffer-pancreas extract was substituted by buffer-hyperglycæmic factor

In case D —

First Diaphragm was incubated in buffer without glucose + hyperglycæmic factor. After two minutes at 25°C. it was washed, then passed to another glass which contained buffer + insulin.

Second Diaphragm was incubated in buffer without glucose + hyperglycæmic factor, and after washing was put into a glass with buffer + glucose without insulin

Third Diaphragm was incubated in buffer without either glucose or factor. Afterwards it was put with buffer + glucose + insulin

Fourth Diaphragm was incubated in buffer without glucose or factor, then settled with buffer + glucose without insulin.

The insulin is expressed in units multiplied by 100 mg. o diaphragm. The glucose uptake induced by the insulin repre- sents the difference between the consumption of glucose of th diaphragm whose buffer contains insulin, and that which doe not contain it. If the consumption of the latter is superior t that which contains insulin we speak of negative consumption

Results

Table I

Influence of Pancreas Extract on the Insulin Glucose-Uptake o the Isolated Diaphragm

Insulin U × 100 mg diaphragm	*Nr exp*	*Glucose-uptake mg × 100 mg diaphragm (Extract)*	*Standard error*	*Glucose uptake— Normal*
0 006	4	0 022	+0 038	0 313
0 042	6	0 203	+0 045	0 387
0 085	4	0 408	+0 081	0 428
0 180	6	0 593	+0 069	0 475

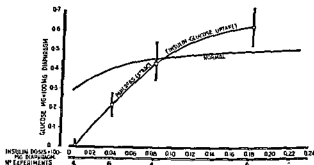


Fig. 1. Influences of pancreatic extract on insulin and glucose-uptake in the isolated rat diaphragm

The examination of the table and the figure shows that the pancreas extract of a normal dog inhibits the action of the insulin when the doses of this hormone are small. After a

Table II

INFLUENCE OF PANCREAS EXTRACT (ALLOXAN DIABETIC DOG) ON THE INSULIN GLUCOSE-UPTAKE OF THE ISOLATED RAT DIAPHRAGM

Insulin U × 100 mg diaphragm	*Nr exp*	*Glucose-uptake mg × 100 mg diaphragm (Extract)*	*Standard error*
0 005	11	−0 303	+0 081
0 030	12	−0 485	+0 080
0 125	7	−0 617	+0 191
0 300	7	−0 732	+0 123

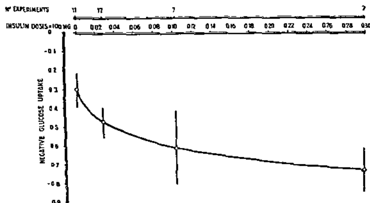


FIG 2 Influence of pancreatic extract (alloxan-diabetic dog) on the glucose-uptake of the isolated rat diaphragm.

The results obtained after the incubation of the diaphragm in a buffer which contains insulin and the pancreatic extract of a dog with alloxan diabetes, point out, as can be judged from the preceding table, that the action of the insulin is inhibited and that, therefore, the glucose-uptake is negative; that is to say, the diaphragm which was put with pancreatic extract and insulin, uses up less glucose than the control diaphragm placed in the same conditions but without insulin. This negative glucose-uptake seems to be accentuated as the dose of insulin increases.

Table III

INFLUENCE OF HYPERGLYCÆMIC FACTOR ON THE INSULIN GLUCOSE-UPTAKE OF THE ISOLATED DIAPHRAGM

Insulin U × 100 mg diaphragm	*Nr exp*	*Glucose-uptake mg × 100 mg*	*Standard error*	*Glucose uptake (normal)*
0 0059	6	+0 037	+0 200	0 310
0 0521	5	+0 122	+0 174	0 390
0 0821	6	−0 170	+0 189	0 420
0 0940	7	−0 409	+0 077	0 430
0 6213	5	+0 022	+0 177	0 626

These results confirm those obtained with alloxanic pancreas extracts. The inhibition which the hyperglycæmic factor produces is clear. However, this diminution of the glucose uptake apparently increases as the dose of insulin increases, if the latter is within the limits 0 005 U–0·6 U.

Table IV

INSULIN GLUCOSE-UPTAKE OF THE RAT DIAPHRAGM PREVIOUSLY INCUBATED WITH HYPERGLYCÆMIC FACTOR

Insulin U × 100 mg diaphragm	*Nr exp*	*Glucose-uptake mg × 100 mg diaphragm*	*Standard error*
0 0152	7	+0 088	±0 0423
0 0784	4	−0 171	±0 0154
0 0983	6	−0 213	±0 0314
0 1417	5	−0 228	±0 0414
0 6195	3	+0 060	±0 0190

These results follow the same line as those obtained in previous experiments. It would seem that the hyperglycæmic factor interferes in some way with the place where the insulin should act and hinders it from producing a positive glucose-uptake.

Discussion

The results set out above are not sufficient for a conclusion to be reached, as it is necessary to extend the number of

experiments, and for other research workers to confirm our findings. However, since we obtained them in experimental conditions which I consider to be correct, the continuity in the results has induced me to present them in order not so much to set down a new fact, as to know your criticism.

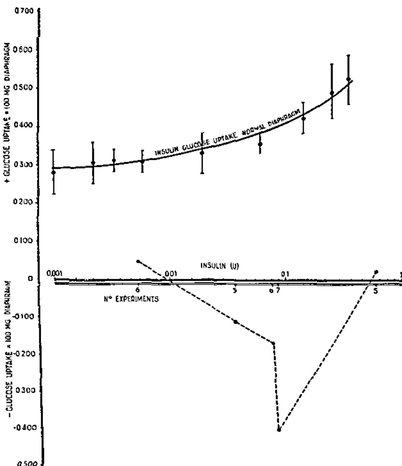


Fig. 3. Influence of hyperglycæmic factor on the insulin and glucose-uptake of the isolated rat diaphragm

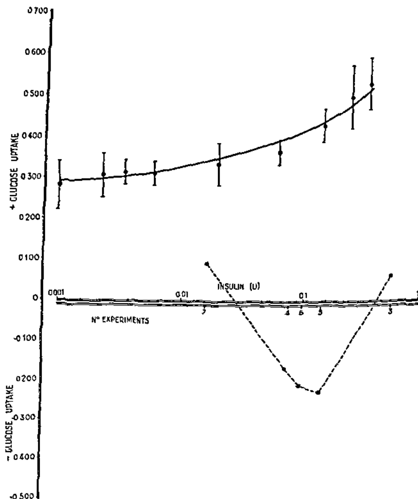


FIG 4. Glucose-uptake induced by insulin in the rat diaphragm previously treated with hyperglycaemic factor.

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DISCUSSION

DE DUVE: In support of Dr Candela's findings, I would like to mention briefly the experiments which were made last year in Professor Bouckaert's laboratory by Tyberghein Rabbits were given 30 units [illegible] epinephrine and the hyperglycæmic factor experiments, did not differ significantly from that of control animals which had been treated for 6 hours with an intravenous infusion of saline and no insulin The muscle glycogen was normal in the animals treated with the hyperglycæmic factor, it was very low in the animals treated with epinephrine Then in another series of experiments, the urinary excretion of urea was followed during the experiments. There was no change in the animals with epinephrine, and there was a trend towards nitrogen retention in the animals receiving hyperglycæmic factor. Now, the main point that I want to make clear is that in these animals that had been subjected to insulin and hyperglycæmic factor during 6 hours, there seemed to be no decrease in the total amount of carbohydrate available, either as glycogen in the liver and muscle, or as blood glucose Nevertheless we have to assume that several grams of glucose would have been utilized under those conditions with insulin [illegible]

[illegible] tissues This would seem to be in line with the experiments mentioned by Dr Candela [illegible]

[illegible] clear-cut effect of the hyperglycæmic factor on isolated diaphragm The little effect we get on glucose uptake is in the direction of an

insulin-like action and I think this is probably due to the small amounts of insulin present in the preparations that we had available. There may be some depression of glycogen storage under these conditions, but the effect that [illegible] clear why our resul [illegible]
We are very sorry [illegible]
basis of other experiments that the hyperglycæmic factor may be liberated under the influence of growth hormone. I might mention that it is very likely indeed that the preparations of glyconeolytic factor at present available may be contaminated with small amounts of insulin.

CAMPBELL: May I ask how you determined the insulin content of the preparations of the hyperglycæmic factor you used?

YOUNG: From the results of diaphragm experiments; that is, you assess the action of glucagon on the isolated diaphragm in terms of insulin. The preparations we used appeared to be contaminated with insulin to about 3 per cent. Also in some experiments which have been carried out by Dr. R. R. Porter at the National Institute of Medical Research, concerning the fractionation of proteins, he was able to show that a contaminating protein was present in our preparations of glucagon which may well be insulin.

BEST: A hyperglycæmic preparation which was alleged to cause [illegible] was discussed

[illegible]

Hoffman (*Proc. Canad physiol Soc., Rev. canad Biol.*, **11**, No. 1, 1952) reported a hyperglycæmic factor isolated from the urine of normal human subjects. This one was tested on the breakdown of glycogen in liver slices.

HUMAN AND EXPERIMENTAL DIABETES

R. D. LAWRENCE

"The twin sisters clinical observation and laboratory experiment have walked, in the field of diabetes, very closely hand in hand" to their mutual stimulation. The latter method is more exact, the former often more initially suggestive. Today I shall deal mainly with the clinical side and try to relate it to the vast new body of experimental facts that are evolving so rapidly and are being freshly discussed here.

In clinical medicine two types of human diabetes have been recognized for a century, and named in France *diabète gras* and *maigre*. Attention became focussed on a pancreatic defect after animal pancreatectomy in 1889 and seemed still more confirmed by the discovery of insulin and its hypoglycæmic action in all diabetics Then the discovery of other endocrine factors, the pituitary, the suprarenal, and possibly the thyroid, proved experimentally the influence of such factors on carbohydrate metabolism, and challenged the whole concept of human diabetes as being due to and explained by the monistic view of primary pancreatic insulin deficiency.

If experimentally the diabetogenic influences of pancreatectomy, of the anterior pituitary hormone, the adreno-glucocortical steroids, perhaps also the adrenal medullary extract and more dubiously the thyroid hormone are granted without doubt, how can we relate this new knowledge to human diabetes? Occasionally, but very rarely, one sees diabetes produced by pancreatic destruction, chronic pancreatitis of different forms, or by hæmochromatosis, rarely an adrenal phæochromocytoma produces diabetes Perhaps a Cushing syndrome may produce a resemblance of diabetes but it is mild and not uniform. Let me turn to the ordinary and vastly more common forms of human diabetes, not yet obviously

related to experimental diabetes, and try to distinguish variations and types of the human disease—because I think they are easily distinguishable clinically and probably are different entities.

Lipoplethoric diabetes, to which I gave this name some two years ago, is the commonest type of diabetes. It occurs in middle age to fat people and is twice as common in women as in men. It is characterized before treatment by a very high blood sugar level (200–400 milligrams per cent), heavy glycosuria, but *no ketosis.* When treated by reducing diets low in total calories without insulin, the "diabetes" disappears when the weight is greatly reduced, and reappears when more food is eaten and weight again increases. I postulated that the diabetes was associated with, and probably due to, obesity and not to insulin deficiency, and we do know from isotope experiments in animals that insulin very quickly guides ingested carbohydrate into stored fat. I said that insulin was quantitatively more lipogenic than glycogenic. I suggested that the overfull fat stores of this type of diabetes could not accept and turn into fat any more of the incoming dietary carbohydrate, with the result that this carbohydrate circulated in excess, the resultant state being called "diabetes." This may be too simple and direct an explanation, and Long has suggested that the great excess of fat with its active metabolism needs much more insulin, which the pancreas fails to produce. I do not believe in his explanation and do not feel sure of my own.

Insulin deficient diabetes is a name I have boldly given to the younger type of diabetic—all children and most adults under 40—and, strangely enough, many who develop diabetes acutely over 70 and even over 80. It is distinguished by loss of weight without previous obesity, and by ketosis of rapidly increasing severity. It should be said that a brief gain of weight often occurs for perhaps a year before acute symptoms. Without insulin they died in coma; with its skilled and continuous use they are normal for years. On clinical grounds alone I have called it insulin-deficient diabetes. This

deficiency I think becomes absolute with no endogenous insulin production.

Lipoatrophic diabetes is another extremely rare type of diabetes of which I described one case fully, or at least as far as my observations went, in 1946. It is characterized primarily by the failure to have fat in any of the usual depots, subcutaneous and retro-peritoneal. This year another similar case was referred to me from Bristol by the kindness of Professor Neale who first recognized the patient's condition, and this removes my slight doubts that this is not a freak but an established syndrome. It is characterized by lack of deposited fat, by a diabetes with intense hyperglycæmia, glycosuria, thirst, etc., but no ketosis, by insulin resistance although insulin in vast doses reduces the sugar, and by an intense hyperlipæmia whenever the glycæmia is high. General health remains good, but an enlarged liver and portal cirrhosis develops and is ultimately lethal. An extremely high metabolic rate is present without any thyrotoxicosis The whole syndrome presents to me an inexplicable picture, but I have presented the explanation that the inability to store fat prevents the usual end-action of insulin and so produces the "diabetic" picture. This presents the opposite state to lipoplethoric diabetics, overloaded with fat deposits, who also cannot readily store circulating ingested carbohydrate so that "diabetes" is also produced. Further speculation to explain both lipo-atrophic and lipo-plethoric diabetes would seem to postulate an unknown enzyme or hormonal factor regulating fat deposition In this I introduce an idea for your discussion and future experimentation on which I have no clear ideas. Professor Gray's department seems to have found a new steroid by paper chromatography but full investigation has been handicapped by lack of the patient's co-operation

Experimental Work

We made an attempt at King's College Hospital to prove these clinical types by animal experiments when Dr. Bornstein joined us with a new technique of plasma insulin estimation

in rats. These rats (ADHA rats) are first made diabetic with alloxan, then hypophysectomized and totally adrenalectomized—a preparation difficult to prepare, maintain and standardize. However, injections of insulin from 1/2,000 to 1/20,000 of a unit of insulin show satisfactory quantitative hypoglycæmic effects. After such standardization, the fundamental test consisted in the injection of 1 ml. of rapidly separated heparinized human plasma from different types of diabetic patients and the observations of any change over one hour in the blood-sugar concentrations of these rats The early results are shown in the following chart —

GROUP I SEVERE, WITH KETOSIS

Patient	1	2	3	4	5
Sex and age	M.32	F 52	F.9	M 28	M.42
Weight (kg)	46	56	25	41	54
Blood-sugar mg %	431	382	406	268	318
Glycosuria	++++	++++	++++	++++	++++
Ketonuria	++++	+	+	+	++
Mean change in blood-sugar .	+1	−3	−3	0	−2
Standard Deviation	±6	±9	±7	±6	±7
Plasma Insulin millunits/ml	0	0	0	0	0

0 = below lower limit of the assay

GROUP II. OBESE, NO KETOSIS

Patient	6	7	8	9	10
Sex and age	M.35	F 47	F 53	F 46	M 57
Weight (kg.)	79	69	75	85	83
Blood-sugar mg %	292	381	312	311	232
Glycosuria	++++	++++	++++	++++	++++
Ketonuria	0	0	0	0	0
Mean change in blood-sugar	−28	−30	−22	−25	−23
Standard Deviation	±19	±9	±6	±8	±8
Plasma Insulin millunits/ml	26	29	19	24	20

Other interesting results are further mentioned and concern the subsequent insulin resistance created by Group I plasma,

insulin resistance, acromegaly and other perplexing problems.

Further results by this technique are now reported and the whole subject brought forward for discussion.

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DISCUSSION

LUKENS. What was the pathology of the liver in those last two cases?

LAWRENCE. Ordinary portal cirrhosis, with no increase in the fat in the one we did examine, the other is still alive

earlier on and then

tic, can't you?

BEST. They get a fatty liver then later the fat disappears from the liver.

GOLDNER. I observed another case of this type a number of years ago. This man too presented a syndrome of hyper-metabolism, absence of subcutaneous fat tissue, enlarged liver and hyperglycæmia with glycosuria. He too was rather resistant to insulin, but his blood lipids as far as I can remember were within physiological limits.

LAWRENCE. Was there absence of hyperlipæmia in states of normal glycæmia?

in c

It

ins

restricted diet. He never went into ketosis or acidosis and was not at all co-operative in our attempts to establish a better control of his diabetes.

LAWRENCE. That was a pity, wasn't it? I've seen a number of these cases with an extremely low carbohydrate diet, which are ketone-free.

a group of patients with diabetes, stops the administration of insulin and keeps them on a carbohydrate free or carbohydrate low diet, ketogenesis occurs. The degree of ketonuria is distributed normally,

caloric diet they get thin and stop passing sugar. When you stop their insulin they don't get ketosis. I can't answer all your questions, really

CONN: I think that all clinicians would agree with Dr. Lawrence about these two types. It was in 1938 that Newburgh and I worked with such patients—so-called middle aged, obese glycosurics. We reduced their weights and got their glucose tolerance tests to normal. Then, as you say, many of them come back having gained weight again and show again a diabetic curve. But I've had an opportunity over the past 10 years to follow some of these patients who have not gained weight and who now have diabetes of the juvenile type. This would support what Dr. Mirsky has suggested, that they may be ends of the same spectrum.

LAWRENCE Yes, I've seen that happen, but on the whole, 70-80 per cent of Group I remain Group I, unless infected, so what end or middle of the spectrum is involved, I don't know.

BEST If it were fashionable to be fat and if you gave this group insulin, would they be happy and get along nicely?

LAWRENCE Yes, if it were fashionable—they would get fatter and fatter, but what about fatty degeneration of the heart?

BEST But they might not get it if they had lots of insulin

LAWRENCE I think it is bad practice, but you have to do it sometimes If you've got a severe pruritus, you must clear them with insulin, but that is not necessary for long term treatment

LONG. We've been interested in the last few years in the metabolism of animals who were made obese by certain types of hypothalamic lesions As you know, if the lesion is suitably placed, the rat will eat two or three times the normal amount of food and will become obese This is also true in other species, particularly in the monkey It is very interesting to see in these animals the development of what Dr. Lawrence calls "lipoplethoric" diabetes Just as soon as they become obese, particularly in the monkey, the glycosuria appears. Rats, as you know, are very difficult to make diabetic, but a certain proportion become diabetic in the sense that they have a high blood sugar, abnormal glucose tolerance curves and glycosuria However, there is one point which bears, I think, on what Dr Mirsky was saying, and that is [illegible]

[illegible]

you begin, [illegible] back on the food intake he initially had, and this time he will have 1 or 2 grams of sugar in the urine As you repeat it, the basal level of the diabetes (if you can call it that), becomes progressively more severe It is certain, therefore, in the experimental animal, that this can

second group?

LAWRENCE. *Might I ask whether those glycosuric rats got ketosis?*

LONG: No, I think they did not. I recall that when in the monkey the glycosuria was very heavy, the ketosis was quite slight. The monkey, of course, is an animal which shows very heavy ketosis on pancreatectomy.

LAWRENCE: Were the islets of Langerhans larger afterwards?

LONG: Well, we didn't study those sufficiently, but with an intact pancreas they usually are

MIRSKY We have studied a number of such patients in terms of the insulin excretion in the urine, and we've also studied patients who are extremely susceptible to ketosis. We could not find any difference in insulin excretion in such patients. It is practically zero in all instances.

SHERLOCK One other point is that in general, I think the first group, i e the fat ones, are the elderly ones and the thin ones who are insulin insufficient, are young It is difficult to believe how one can go into the other. The fat ones are the elderly.

LAWRENCE I meant to refer to Wrenshall's publication in Best's department, about the extractable insulin from the pancreas He showed a clear division of maturity group and growth group The

RESPONSE OF THE LIVER TO INSULIN; HEPATIC VEIN CATHETERIZATION STUDIES IN MAN

A. G. BEARN, BARBARA H BILLING and SHEILA SHERLOCK

HEPATIC vein catheterization can be used in the measurement of the output of glucose from the liver. In 1944, Warren and Brannon introduced a safe method of catheterizing the hepatic vein in man. A radio-opaque "Nylon" catheter is inserted into an antecubital vein, and is passed under fluoroscopic control via the superior vena cava into the right auricle. The catheter is then directed posteriorly to enter the upper part of the inferior vena cava. Rotation anteriorly and to the right enables the catheter to enter a branch of the right hepatic vein and hence be inserted deeply into the right lobe of the liver (Sherlock *et al*, 1950)

The blood-flow through the liver is determined by the bromsulphalein extraction method (Bradley *et al.*, 1945). The glucose concentrations in hepatic venous and capillary blood are estimated The glucose content of the capillary blood is assumed to be the same as that of arterial blood. The output of glucose from the liver can now be calculated by the formula: Hepatic glucose output = (hepatic venous glucose concentration − capillary glucose concentration) × hepatic blood-flow.

The difference in concentration of glucose in the hepatic vein and systemic artery cannot be ascribed solely to the liver. The results therefore indicate changes in the entire splanchnic area (liver, spleen, and intestines) and not in the liver alone. In the fasting state, however, the concentration of glucose in the portal vein is the same as that in a systemic artery (Cherry and Crandall, 1937; Sherlock and Walshe, 1946), and

little error is introduced by referring to hepatic metabolic changes rather than to splanchnic changes.

Recently, Bondy (1952) has shown very great spontaneous variations in the glucose content of the hepatic venous blood and doubts the validity of estimates of splanchnic glucose balance for any period other than the actual time of sampling. We have taken samples from the hepatic vein at 5-minute intervals over two separate one hour periods in two normal subjects, and although there are slight variations in the glucose concentrations they are never so great as to change the general trend of results. Moreover, taking mean values for a large group of subjects will tend to smooth out these variations.

In all the 15 normal subjects studied, insulin 0 1 unit/Kg. injected intravenously resulted in a fall in the concentration of glucose in the hepatic venous blood This occurred within two minutes of giving the insulin Identical results were obtained with "novo-insulin," a preparation known to be free of glycogenolytic factors The effects of the "Boots" insulin which we used are therefore not due to any glycogenolytic factors it may contain. The capillary glucose concentration also fell, but less rapidly than that in the hepatic vein. Five minutes after the administration of insulin the mean differences in the concentrations ($H_{GL}-C_{GL}$) had dropped from 14·5 to 6 4 mg. per 100 ml blood This change was the expression of an immediate decrease in hepatic glucose output which remained below the normal value for 30 minutes (Fig 1). In 9 of 12 subjects, the difference ($H_{GL}-C_{GL}$) was a negative quantity in one or more of the samples taken at 5, 15 or 30 minutes after the insulin Insulin can therefore not only cause a reduction in the output of glucose from the liver but also a withdrawal of glucose by the liver from the circulating blood. Minimum values for capillary glucose concentration (mean 38 ± 2 6 mg., S E., per 100 ml.) were observed in all subjects 30 minutes after the insulin had been given. At this time there were usually clinical signs of hypoglycæmia, shown by sweating, drowsiness, pallor, increased pulse rate and a fall in diastolic blood pressure The hepatic glucose output had,

however, already begun to return to normal and at 45 minutes was, except in one observation, well above basal levels. This increased output was responsible for the increase in capillary glucose concentration, although 100 minutes after the insulin this had not reached the normal value. There was no constant relationship between the diminution in hepatic glucose output following insulin and the increased hepatic glucose output during the recovery phase.

The recovery of the blood glucose level after insulin is probably due to more than one factor. Hypoglycæmia results in the liberation of adrenaline. In normal man the infusion of adrenaline increases the hepatic blood flow, hepatic glucose output, peripheral and hepatic venous lactic acid concentration and the splanchnic oxygen consumption (Bearn *et al.*, 1951). All these results were observed in normal subjects after insulin administration and are probably due to the release of adrenaline. Moreover, diabetic subjects, in whom there was no hypoglycæmia, did not show the hepatic blood flow, hepatic glucose output, lactic acid and splanchnic oxygen consumption changes which we have attributed to adrenaline.

When adrenaline is given to normal subjects the hepatic glucose output increases immediately and at the same time as the rise in hepatic blood-flow and blood lactic acid (Bearn *et al.*, 1951). The rise in hepatic glucose output after insulin, however, precedes these other changes (Fig 1). The restoration of the blood glucose is therefore not due only to adrenaline. When all the insulin administered is destroyed, the hepatic glucose output would presumably return to the pre-insulin level. It seems unlikely that all the insulin would have disappeared within thirty minutes of injection, for diabetic subjects, in whom hypoglycæmia does not occur, show a depression of hepatic glucose output for an hour after insulin. Moreover, in diabetic subjects the small dose of insulin does not cause hypoglycæmic symptoms and presumably no adrenaline release, and yet the blood glucose is eventually restored to its pre-insulin values. Hexamethonium compounds, by blocking sympathetic impulses to the liver and adrenal

medulla, are said to increase the degree of hypoglycæmia after insulin (Laurence and Stacey, 1952). Preliminary observations using hepatic vein catheterization have failed to confirm this

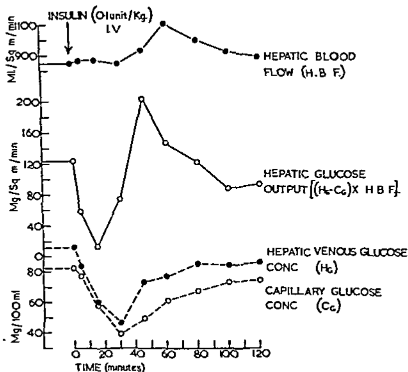


FIG. 1 The effect of insulin on hepatic blood flow and hepatic glucose output in normal subjects. (*Clin Sci*, 1952, p 152.)

finding and suggest that the blood glucose returns just as rapidly when hexamethonium bromide is given with the insulin as when insulin is given alone (Paton, Reynolds and Sherlock, 1952). This again suggests that recovery of the blood glucose concentration after hypoglycæmia can occur without

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curve of diminished hepatic output of glucose after insulin (Fig 2). This area measures the difference in rate of output of glucose over the period of thirty minutes, and indicates an absolute amount of glucose, measured in grams. It has been termed the "diminished hepatic output of glucose"

The difference between the total amount of glucose removed from the circulation, less urinary glucose loss and diminution of hepatic output, will indicate the peripheral utilization of glucose after insulin. The total amount of glucose removed

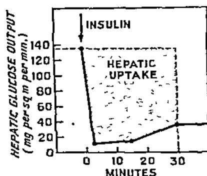


Fig 2 [illegible] shaded area represents the amount in grammes

can be calculated from the fall in capillary glucose concentration if the space into which glucose diffuses is known. Recent work in the rabbit using glucose containing radio-active carbon (C^{14}) suggests that the glucose space is 30 per cent of the body weight and does not change significantly after insulin (Drury and Wick, 1951; Wick *et al.*, 1950). Using this assumption the total amount of glucose disappearing from the "glucose space" after insulin can be calculated from the drop in the capillary glucose concentration. Subtraction of the diminished hepatic glucose output and the urinary glucose loss from this figure gives an approximate value for the increased peripheral

adrenaline production. Other factors capable of increasing hepatic glucose output must be considered. The α cell hormone prepared from the pancreatic islets is glycæmic and acts directly on the liver (Pincus, 1950). It is not known whether this substance is released when the blood glucose level is lowered. Finally the liver may possess an inherent homeostatic property of maintaining a normal blood glucose level (Soskin *et al.*, 1938). This seems the most likely explanation but has not been proved.

There is no doubt that insulin diminishes the output of glucose from the liver. This is at variance with frequent animal experiments where insulin has lowered liver glycogen. As this depletion occurs in the adrenalectomized preparation, it cannot be due to adrenaline secreted in response to hypoglycæmia. Similarly if glucose is given with the insulin to prevent hypoglycæmia, liver glycogen is still diminished (Bridge, 1938) These findings support the suggestion that the primary effect of insulin is to increase the disposal of glucose in the peripheral tissues, and that the output of glucose from the liver rises secondarily to satisfy the demand of the peripheral tissues Our results, moreover, are supported by *in vitro* studies showing that insulin inhibits glycolysis in the dog's liver (Soskin, 1941). Bouckaert and de Duve (1947) also stress the importance of the liver in lowering the blood glucose level after insulin The differences between the older work and the present results might be related to the type of insulin employed. Until recently many commercial preparations of insulin contained a glycogenolytic factor. The insulin used in the present work did not contain this factor

It would be interesting to quantitate the extent of the depression of the hepatic glucose output after insulin injection. This can be attempted by measuring the depression of the hepatic glucose output for thirty minutes after the insulin, i.e., before the onset of hypoglycæmia. This depression can then be expressed in terms of an absolute amount of glucose. This is done by measuring with a planimeter the area of the curve between the fasting hepatic glucose output and the

having values of between 2 g. and 6 g. Diabetic subjects show a wide scatter of results. Thirty per cent of 33 have a hepatic glucose uptake of less than 2 g. and 37 per cent greater than 6 g. (Fig. 3).

This difference in the hepatic response to insulin enabled *the diabetics to be classified into two different groups. In the*

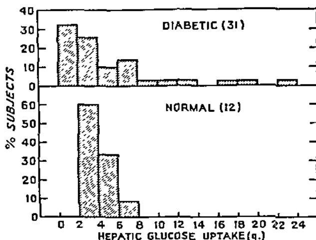


FIG 3 Range of hepatic uptake of glucose in 12 normal subjects and in 31 diabetics 30 minutes after intravenous insulin. (*Lancet*, 1951, ii, 699.)

hepatic insulin-sensitive group the hepatic glucose uptake is greater than the upper range for normal subjects. The hepatic-insensitive group have values less than the lower limit for normal subjects. The hepatic insulin sensitivity can be correlated with the clinical features of the disease and with the histology of the sections of liver obtained by biopsy.

The *hepatic-sensitive diabetics* were underweight and in the young age-group They manifested an unstable type of diabetes, readily becoming ketotic without insulin and hypoglycæmic with but slight insulin excess. Fundal changes,

utilization of glucose after insulin (Table I). These results show that the liver plays an important part but cannot be solely responsible for the fall in blood glucose level after insulin. This is in keeping with the observation that insulin

Table I

MEAN DIMINUTION IN HEPATIC GLUCOSE OUTPUT AND MEAN CALCULATED INCREASE IN PERIPHERAL UTILIZATION OF GLUCOSE FOR 30 MINUTES AFTER INSULIN

Subjects	*No*	*Total glucose removed from glucose space* g (T)*	*Diminished hepatic output of glucose g (H)*	*Peripheral utilization of glucose g (T-H)*
Normal	12	10 3 (6 1–13 9)	4 1 (2 5–6 6)	6 2 (3 1–10 0)
Diabetic	27	12 6 (2 8–28 6)	4 2 (0 3–23 4)	8 4 (−7 4–21 8)

The decrease in capillary glucose concentration in mg /100 ml whole blood has been converted to the decrease per 100 ml blood water It has been assumed that the decrease occurs in a "glucose space" equal to 30 per cent body weight.

*Urinary loss of glucose has been deducted

increases muscle glycogen (Gemmill, 1940), and increases the rate of oxidation of carbohydrate in tissues (Villee and Hastings, 1949).

The 43 diabetic subjects who were studied were unselected; their ages were from 15 to 70 years, and the majority were aged 45–55 years. Twenty-six of them had never received insulin, while the remainder had had no insulin for at least forty-eight hours prior to the observation. They showed greater fluctuations in fasting hepatic glucose output than did the normal subjects. Allowing for urinary loss of glucose the value was not significantly increased and the evidence, although not conclusive, strongly suggests that the over-production of glucose from the liver is not the reason for the high blood glucose values of diabetic patients.

In normal subjects the diminished hepatic output of glucose after insulin is very constant, 90 per cent of the 12 studied

Summary

The hepatic vein catheterization technique has been used to study the effect of insulin on the liver in 15 normal and 43 diabetic subjects.

Insulin (0·1 unit/kg. i.v.) results in an immediate drop in the hepatic glucose output in both normal subjects and diabetics.

When normal subjects become hypoglycæmic they show an increase in hepatic glucose output, in hepatic blood flow, in venous lactic acid concentration and in splanchnic oxygen consumption. These events are probably due to the release of adrenaline.

In normal subjects the restoration of the blood glucose concentration to normal after insulin is not due to adrenaline alone Other possible factors are discussed.

The extent of the depression of the hepatic glucose output and the increased peripheral utilization of glucose after insulin have been calculated. Both factors play a part in lowering the capillary glucose concentration.

In normal subjects the fall in hepatic glucose output after insulin is fairly constant. In diabetic subjects the fall in output is variable and enables the diabetics to be divided into hepatic-sensitive and hepatic-insensitive types.

In hepatic-sensitive diabetics administration of insulin results in a greater fall than normal in the hepatic glucose output. The patients are young and thin, and readily go into ketosis. Sections from aspiration biopsy of the liver show no histological abnormalities.

In hepatic-insensitive diabetics insulin results in a smaller fall than normal in the hepatic glucose output. The patients are middle-aged and obese, and rarely go into ketosis. Sections from aspiration biopsy of the liver show fatty change in the liver.

Hepatic-sensitive diabetics when in severe ketosis become hepatic-insensitive. Sections of liver show no remarkable histological change.

cataract, peripheral vascular disease, and neuritis were unusual in this group.

Sections from an aspiration biopsy of the liver showed remarkably normal histology; fatty change was not seen. The glycogen content of the liver was qualitatively normal. Quantitative analyses of liver-glycogen in similar subjects has also been shown to be normal (Hildes *et al.*, 1949)

The *hepatic-insensitive diabetics* can be subdivided into the "elderly type" and patients in severe ketosis.

The elderly type were usually more than 50 years old; they were often obese. The disease was more stable; ketosis did not occur and hypo-glycæmic symptoms were induced with difficulty. In many instances the diabetes was symptomless and only discovered at a routine medical examination. Fundal changes and cataract, peripheral vascular disease, and neuritis were *common complications*.

Sections from an aspiration biopsy of the liver usually showed fatty change A significant negative correlation could be established between the hepatic uptake of glucose after insulin and the extent of the fatty change in the liver (Sherlock *et al.*, 1951) Fibrosis was not seen. The glycogen content of the liver was qualitatively normal

The *severely ketotic type*. Three diabetic patients were studied while in severe ketosis (alkali reserve less than 10 m. eq per litre). These patients were clinically of the juvenile hepatic-sensitive type. While in severe acidosis, however, hepatic vein catheterization studies showed them to be hepatic insulin-insensitive. When the diabetes was controlled they reverted to a sensitive state Aspiration biopsies of the liver showed normal histology, and the glycogen content of the liver was well preserved even in specimens taken at the height of ketosis.

Finally it must be emphasized that there is inevitably some overlap between the two hepatic and clinical types of diabetes. Although, apart from ketosis, a young diabetic is rarely hepatic-insensitive, an occasional elderly thin diabetic with cardiovascular complications may be hepatic insulin-sensitive.

a normal rate. At that stage, glucose does not need to be over-produced by the liver

DE DUVE: I would like to ask Dr. Sherlock whether the measurements on diabetics were made on untreated diabetics with high blood sugars?

SHERLOCK Yes; 26 had never had insulin and the remainder had been off insulin for 48 hours.

SHERLOCK It's quite possible.

SHERLOCK. I believe that is quite possible.

C F. CORI I think the position is that insulin depresses glucose output by the liver. In the diabetic liver without insulin, the response to blood sugar changes may not be the same as in the normal liver

BEST There's going to be a little difficulty if we can't depress the blood sugar without giving insulin

GOLDNER From a rather small number of experiments on hepatic glucose output in men by means of hepatic vein catheterization, I can confirm Dr Sherlock's statement that under basal conditions the blood sugar level in the hepatic vein is rather constant even for prolonged periods of time, such as 2–3 hours. Spontaneous fluctuations of the curve which were seen occasionally seemed in each instance to be attributable to excitation of the patient or other disturbances of the basal state. Moreover, and again in confirmation of Dr Sherlock's finding, we saw no significant difference in the hepatic glucose output of the diabetic without insulin as compared with the normal subject

LONG Dr Sherlock, have you tested the nitrogen exchange across these livers?

SHERLOCK Well, his hepatic blood flow figures with the urea method agree well with those obtained by the B S P. method He was however only able to make a few measurements under basal conditions He wasn't attempting to continue over two hours with multiple sampling, because the urea analysis with the needed accuracy requires large blood samples You'd just have an exsanguinated patient if sampling was repeated twelve times

G CORI It seems to me that Dr Sherlock has given the *coup de grâce* to the wrong theory which has influenced physiological work, not for the better, for many years

CAMPBELL I think Dr. Long showed that when the blood sugar

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DISCUSSION

BEST: You contend that insulin decreases the sugar output of the liver but the sugar output is not increased in the untreated diabetic? Is this the conclusion you came to originally?

SHERLOCK: Yes, I haven't changed my mind

BEST I was under the impression that you thought there was an increased production in the diabetic

SHERLOCK: No, the only ones that may have an increased production —and I have not really studied enough patients to say definitely—are the very severely acidotic diabetics in coma These are a very tiny percentage of diabetics The ordinary diabetic, of whatever type, does

THE INSULINASE AND INSULINASE-INHIBITOR ACTIVITY OF THE LIVER

I. ARTHUR MIRSKY

The metabolic derangement of diabetes mellitus in man or animals is due to an insufficiency of insulin relative to the requirements by the tissues. With pancreatic insufficiency, whether surgically or pharmacologically induced, the availability of insulin is reduced, while the requirements for insulin remain essentially unchanged. In only a relatively small proportion of instances, however, can the diabetes mellitus of man be attributed solely to a diminution in the production of insulin (Mirsky, 1945). Consequently, it has been proposed that the human syndrome is due in large measure to an increase in the requirements for insulin

That the contra-insulin effects of the hormones of the anterior pituitary, adrenal cortical, and other glands may increase the insulin requirements is well established. Yet, the syndrome resulting from the excessive secretion or the administration of these hormones differs markedly from the diabetes mellitus which occurs in approximately 1 per cent of the population. It would appear, therefore, that some other factor or combination of factors may be responsible for the increase in the peripheral requirements for insulin which appears to be responsible for the syndrome of diabetes mellitus in man.

Since it is generally acknowledged that insulin is destroyed rapidly in the intact organism (Greeley, 1940, Mirsky, Podore, Wachman and Broh-Kahn, 1948) we considered it essential to obtain information concerning the mechanism whereby insulin is destroyed in the tissues Towards that end, the ability of tissue extracts and slices to inactivate insulin during *in vitro* incubation was studied

level is decreased there is a very sharp reduction in the ascorbic acid content of the adrenal gland, indicating a secretion of the adrenal cortical steroids in response to the hypoglycæmia. I wondered if you had coupled that with the possible explanations for the allied compensatory rise in blood sugar.

SHERLOCK Yes, we have, and that was why I asked Dr. Long the other day how long he thought it would take for adrenal steroids to raise liver glycogen It is so quick. You see, the insulin action only reduces hepatic glucose output for half an hour, then it recovers, and I thought this recovery was probably too rapid to be due to adrenal cortical steroids

LONG The release of ACTH is certainly very rapid Whatever views you may take on the nature of the mechanism responsible, I think most people agree that it can be released very rapidly and in probably a matter of seconds

SHERLOCK How long would it take to act on the liver?

LONG: That is a very difficult question to answer under the circumstances such as this This is what was at the back of my mind when I asked about the nitrogen exchange

CONN: Have you tried giving intravenous cortisone in experiments of this kind? One might answer that problem quickly

SHERLOCK I think we'll probably have to do that.

LAWRENCE: It seems to me that your diabetics are very long-suffering, aren't they?

SHERLOCK Yes. I think I ought just to mention the safety of this technique Hepatic vein catheterization is an extremely safe technique It is much safer than aspiration liver biopsy and much safer than cardiac catheterization performed for the investigation of patients with heart diseases. The catheter for hepatic vein work only enters the right auricle—all the dangers of cardiac catheterization arise from introducing a catheter into the right ventricle and pulmonary artery We are avoiding the danger spots Hepatic vein catheterization is a very justifiable and safe procedure I personally, in an experience of at least 200, have never had any difficulties or complications, and I don't know of any other group which has

the liver. It is of some importance to note that brain and blood were found to be poorest in insulinase activity, even though these tissues are known to be quite rich in proteolytic and peptidase activity (Table I). In general, these results substantiated the findings of Schmidt and Saatchian (1929) with

Table I

DISTRIBUTION AND ACTIVITY OF INSULIN INACTIVATING SYSTEM IN RAT TISSUE EXTRACTS

Incubation for 60 minutes at pH 7 5 and 37°C. Organ Extracts prepared in usual manner Washed blood cells resuspended in saline in original concentration. 1 ml of insulin containing 8 units.

Tissue	*Pooled percentages of Initial Blood Sugar*
Liver	211
Kidney	150
Muscle	112
Brain	84
Whole Blood	92
Plasma	55
Cells	91

reference to the insulin inactivating properties of brei prepared from various tissues of the rabbit.

The system responsible for the inactivation of insulin appears to have the properties of an enzyme. It is heat-labile, being destroyed by exposure to 80°C for 10 minutes. Maximum activity during incubation occurs at reactions between pH 6·5 and pH 9 5. At pH 7·5 the rate of insulin inactivation increases appreciably with a rise in the temperature of incubation from 0° to 37°C. A further increase in the incubation temperature to 55°C. fails to affect the rate of inactivation of insulin. At pH 7·5 and 37°C., the destruction of insulin proceeds at a fairly rapid rate which gradually decreases toward the end of the incubation period.

In rat liver extracts, the insulinase activity was almost quantitatively removed from solution by precipitation of the proteins, either through adjustment of the reaction to pH 5 or by half-saturation with ammonium sulphate at pH 6·7.

The present report summarizes the evidence that the liver, and to a lesser degree other tissues, contains a system which is capable of inactivating insulin as well as a factor which inhibits the action of this system. For the sake of convenience, the insulin-inactivating system is referred to as "insulinase" while the factor which prevents its action is referred to as "insulinase-inhibitor" (Mirsky and Broh-Kahn, 1949, Broh-Kahn and Mirsky, 1949; Mirsky, Simkin and Broh-Kahn, 1950).

Unless otherwise indicated, extracts were prepared from tissues by homogenizing in a blendor, or by the Potter and Elvehjem (1936) technique with three volumes of cold water. After centrifugation at high speed, the filtered supernatant fluids were adjusted to pH 7 5 and tested for their ability to inactivate insulin. In the standard procedure, 1 ml. of extract together with 1 ml. of solution containing 8 units of insulin were incubated at 37° for from 30 to 60 minutes. After completion of the incubation, 1 ml of the mixture was injected intravenously into a fasted rabbit. Blood samples were drawn prior to and at 30 and 60 minutes after injection. If no destruction of insulin had occurred, the volume injected would contain four units of insulin which is sufficient to produce a profound hypoglycæmia in the rabbit. The change in the blood sugar concentration was expressed as the percentage of the pre-injection blood sugar level The sum of the percentages of the 30 and 60 minute blood samples was designated as the "pooled percentage of initial blood sugar" which in turn was utilized as a semiquantitative expression of the activity. If no insulin were destroyed during incubation, the "pooled percentage" would be very low, whereas if all the insulin were destroyed during the period of incubation, the blood sugar concentration would remain unchanged and the "pooled percentage" would equal 200.

Systems capable of inactivating insulin during *in vitro* incubation were found to be distributed widely, but in all species studied (rabbit, steer, chicken, man and rat), the highest activity per gram of tissue was found in extracts of

Table III

EFFECT OF INHIBITORS ON DESTRUCTION OF INSULIN BY RAT LIVER EXTRACTS

Incubation for 30 min. at pH 7 5 and 37°C Standard procedure as described in text Incubation mixtures originally contained 8 units of insulin

Inhibitor	*Final concentration*	*Pooled percentages of initial blood sugar*
None		195
$CuSO_4$	0 0005 M	91
$ZnSO_4$	0 0005 M	118
Iodoacetate	0 001 M	117
Iodosobenzoate	0 001 M	84
Soybean inhibitor	0 45 mg	212
Normal human serum	0 9 ml	193

activity These observations suggest that the participation of a sulphhydryl group in the activity of insulinase is essential. Attempts to reactivate the insulinase with cysteine, however, were unsuccessful In confirmation of the aforementioned, DeBarbieri and Grassi (1951) demonstrated that the oxidation of -SH groups resulted in an inactivation of insulinase activity

To determine whether the inactivation of insulin by liver extracts is due to some non-specific proteolytic enzyme system, the effect of such extracts on denatured hæmoglobin at pH 7 5 was measured. No appreciable activity was noted even with incubation periods as long as 6 hours. Furthermore, the addition of the relatively non-specific proteinase inhibitors of soya bean and of human serum did not appreciably decrease the insulinase activity of the extracts On the other hand, the proteolytic activity of crude liver extracts at pH 3 5 was quite significant. Concomitant measurements of the insulinase activity at pH 7·5 and the proteolytic activity at pH 3 5 revealed that whereas the former may diminish markedly even when stored at 5°C., the proteolytic activity of the same extract remains unchanged.

Working with homogenates of liver as well as with extracts prepared by fractionation with acetone and sodium sulphate at pH 5·2, De Barbieri and Grassi (1951) demonstrated a

During precipitation, only moderate destruction of the active proteins occurred and much of the activity was recovered from the washed precipitates by redissolving the latter and adjusting to *p*H 7·5–8·0.

Dialysis of rat liver extracts for three hours resulted in a decrease in insulinase activity. The addition of manganese and magnesium ions to the non-dialysable residue resulted in partial restoration of the activity. Other ions that were tested were found to be ineffective.

The distribution of insulinase activity *in the components* of homogenates was determined. Towards that end, livers were homogenized in a Potter-Elvehjem homogenizer with three volumes of 0·88 M sucrose and fractionated in accordance with the procedure of Hogeboom, Schneider, and Pallade (1948). Table II reveals that the insulinase activity is confined

Table II

DISTRIBUTION OF INSULINASE IN RAT LIVER HOMOGENATES PREPARED IN 0 88 M SUCROSE

Preparation	*Pooled Percentage of Initial Blood Sugar*	
	I	*II*
Homogenate	239	205
Nuclei and residued cells	161	
Mitochondria	85	95
Supernatant after 20 min at 18,000 g	113	170

principally to the supernatant after removal of cellular debris and mitochondria

The inactivation of insulin by liver extracts could be inhibited by a number of substances (Table III). Thus, the *in vitro* addition of small amounts of copper, zinc, and certain other heavy metals resulted in almost complete suppression of the insulinase activity, whereas salts of magnesium, manganese, sodium and calcium, failed to influence the activity of the system Moreover, the addition of 10^{-3} M iodoacetate and iodosobenzoate resulted in a marked inhibition of insulinase

even in the presence of acid, resulted in a destruction of the inhibitor. Dialysis in the cold led to rapid loss of activity.

Some degree of purification of the insulinase-inhibitor was obtained by the method used by Grollman and Woods (1949). With this procedure, an appreciable quantity of the inhibitor activity of crude trichloracetic acid extracts of beef liver could be adsorbed on charcoal, eluted with glacial acetic acid and precipitated with a mixture of absolute alcohol and petroleum ether. Although much of the activity present in the crude extract is lost during the process of purification, an approximately four-fold increase in activity is obtained

In order to determine whether or not the action of the insulinase-inhibitor was due to a non-specific inhibition of some proteolytic activity, its effect on the proteolytic activity of crystalline pancreatic trypsin, crystalline chymotrypsin, crystalline pepsin, plasmin, and liver extracts was determined as exemplified in Table IV. In no instance was an appreciable

Table IV

LACK OF EFFECT OF INSULINASE INHIBITOR ON TRYPSIN AND HUMAN PLASMIN ACTIVITY

Trypsin and plasmin activities estimated from amount of acid-soluble "tyrosine-like" material liberated during incubation for 60 min with a hæmoglobin substrate at 37°C and pH 7 5 The reaction mixtures consisted of

	"Tyrosine" liberated µg /ml
Trypsin	1070
Trypsin + inhibitor	1113
Plasmin	285
Plasmin + inhibitor	276

inhibition of proteolytic activity produced in the presence of insulinase-inhibitor Such observations, together with those discussed above, again emphasize the lack of identity between insulinase and the more common proteinases.

parallelism between insulinase activity and the proteolytic activity at pH 7·5 and 3·5. They noted also that when an -SH oxidizing agent was added to an extract, both insulinase and proteolytic activities were inhibited. Consequently, these investigators concluded that insulinase is a peptidase in which thiol groups play an essential rôle. Such a conclusion, however, is not in accord with the data cited above nor with the observation that whereas homogenates of brain and hæmolysates of erythrocytes are rich in proteinase and in peptidase activity, they show minimal insulinase activity.

During the course of attempts to purify the system responsible for the *in vitro* inactivation of insulin by liver homogenates and extracts, it was noted that the insulinase activity could be precipitated almost quantitatively with acetone. Such precipitates, however, when redissolved in water and reconstituted to the same volume as that of the extracts from which they had been prepared, were invariably more active than the crude material. Such observations suggested the presence of an insulinase-inhibitor in the crude extract.

The actual presence of an inhibitor in the acetone-soluble supernatant solution was readily demonstrated (Mirsky, Simkin, and Broh-Kahn, 1950). After acetone was removed by distillation *in vacuo*, and the residue extracted with water, clear filtrates of the aqueous phase were found to contain the inhibitor, as was demonstrated by their effect in suppressing the activity of potent preparations of insulinase Within certain limits, the degree of inhibition produced was roughly proportional to the quantity of inhibitor added

The *insulinase-inhibitor* was found to be quite soluble in water, acetone, 95 per cent ethyl alcohol, hydrochloric acid, and trichloracetic acid, but insoluble in ethyl and petroleum ethers. In acid solutions, the inhibitor was quite stable to heat In solutions adjusted to pH 0·5, the inhibitor was not appreciably affected by autoclaving for as long as 30 minutes at 15 lb. pressure. Similar treatment at pH 8·0 resulted in considerable loss At pH 11·7, rapid destruction of the inhibitor occurred merely by standing at room temperature. Ashing,

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remains so for as long as 162 hours. When animals which had been fasted for 96 hours are permitted to feed *ad libitum* on stock laboratory rations for 24 hours, the insulinase activity of extracts of their livers is restored to the range of values obtained from unfasted rats.

In view of the preceding, the effects of a high-carbohydrate, a high-fat, and of a balanced diet upon the restoration of the insulinase activity of fasted rats were studied It was demonstrated that the restoration of insulinase activity per unit weight of liver of previously fasted rats occurs equally well with all types of diet. When the insulinase activity was calculated on the basis of the total weight of the liver rather than as per unit weight of liver, it became apparent that a high-carbohydrate diet produces a greater increase in the total insulinase activity of the liver than do the other diets. Furthermore, since the ratio of the liver weight to the body weight was highest in the rats re-fed with the high-carbohydrate diet, such rats contain more liver insulinase per unit body weight.

The assay of the insulinase activity of extracts yields information concerning the potential activity released after cellular disintegration and undoubtedly does not represent the actual "working" activity of the cell prior to homogenization Consequently, the destruction of insulin by slices of rat liver was studied (Mirsky and Perisutti, 1952) Slices of approximately 1 cm.^{2} in size were prepared and incubated in 3 ml of phosphate buffer containing 3 units of insulin. At the end of the incubation period, 1 5 ml. of the incubation mixture, representing an original content of 1 5 units of insulin, were removed and administered intravenously to a rabbit. The slices were dried overnight to constant weight The insulinase activity was then estimated as the "pooled percentage of initial blood sugar" and weight of the slices was expressed in terms of the dry weight.

As could be anticipated, the insulinase activity of rat liver slices is much smaller than that of an homogenate or extract of an equivalent amount of liver. Whereas from 8 to 12 units of insulin are inactivated after incubation for 30 minutes by

In order to evaluate the physiological significance of insulinase, the influence of various factors, known to be associated with changes in carbohydrate metabolism, on the insulinase activity of liver extracts was investigated. For such purpose, the effects of fasting (Broh-Kahn and Mirsky, 1949), and of

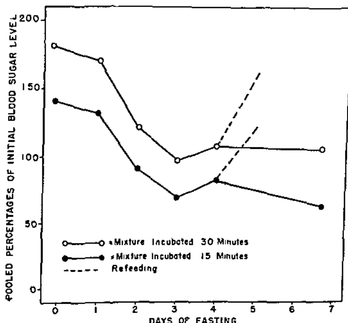


FIG. 1. The effect of fasting and refeeding on insulinase activity of rat livers Incubation at pH 7 5 and 37°C for the designated time (Broh-Kahn and Mirsky, 1949)

refeeding with various types of diets (Broh-Kahn, Simkin and Mirsky, 1950) on the insulinase activity of liver extracts were determined.

Fig 1 illustrates the effect of fasting on the insulinase activity of extracts of comparable quantities of liver. It is evident that fasting is associated with a marked reduction in the insulinase activity of liver extracts. This reduction approaches statistical significance at the end of 48 hours of fasting and

of the liver slices revealed a highly significant relation in spite of the scatter. Thus, it is apparent that with increasing quantities of liver, there is an increase in insulinase activity. It is pertinent to note that when insulin was absent from the incu-

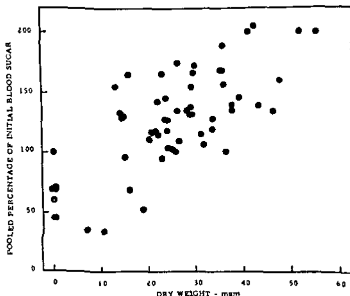


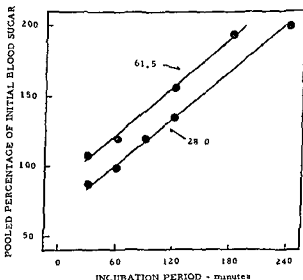
FIG 3 The relation between the quantity of liver slices of fed rats and the inactivation of insulin Slices from livers of fed animals were incubated in 3 ml phosphate buffer containing 3 units of insulin at pH 7 5 and 37°C for 60 minutes Rabbits injected with 1 5 ml of incubation mixture

bation mixture, the subsequent injection of an aliquot did not affect the blood sugar of rabbits.

Since fasting results in marked diminution in the insulinase activity of liver extracts, it became pertinent to determine the influence of fasting on the activity of liver slices Fig. 4 illustrates the data obtained with slices from livers of rats fasted 72 hours. Analysis of the covariance revealed that the activity of slices from livers of fasted rats is significantly greater than of those from fed rats ($P < 0{\cdot}001$)

an homogenate of 300 mg. liver, only about 2 units are inactivated in the same time by an equivalent quantity of slices.

The linear relation between the duration of the incubation period and the destruction of insulin by liver slices is illustrated in Fig. 2. It is apparent also that the rate of insulinase



[illegible] on the inactivation

activity, as indicated by the regression of "pooled percentage" on time of incubation, is the same for slices weighing approximately 28 mg. and for slices of approximately 61Y5 mg., although the quantity of insulin destroyed at any particular time interval is greater with the larger quantity of liver.

The relation between insulinase activity and the quantity of liver slices is illustrated in Fig. 3. Calculation of the linear regression co-efficient of insulinase activity on the dry weight

As with liver extracts, the insulinase activity of slices of livers from fed rats is suppressed by the addition of liver insulinase-inhibitor to the incubation mixture (Fig. 5). Analysis of the covariance revealed that the effect of the inhibitor on

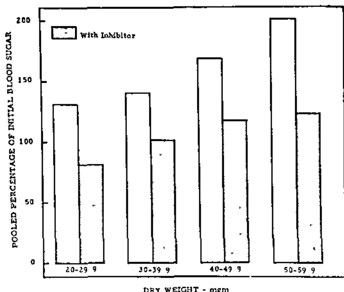


Fig. 5. The effect of [illegible] inhibitor on the [illegible]

the insulinase activity of different quantities of liver slices is highly significant ($P < 0.001$)

The physiological significance of insulinase and insulinase-inhibitor is difficult to establish at the present time. The presence of these factors, however, supports the hypothesis that an insufficiency of insulin may result from an increased destruction of insulin, either in consequence of an increase in insulinase activity or of a decrease in the availability of the insulinase-inhibitor.

The discrepancy between the effect of fasting on the insulinase activity of extracts and of slices suggested that although the total potential insulinase activity may be diminished, the insulinase-inhibitor may be diminished to a greater degree

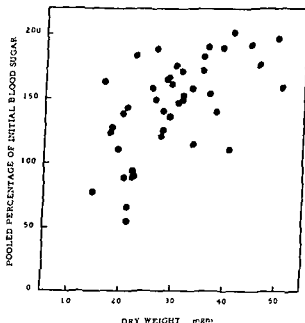


FIG 4 The relation between the quantity of liver slices of fasted rats and the inactivation of insulin. Slices from livers of 72 hour fasted animals were incubated in 3 ml. phosphate buffer containing 3 units of insulin at pH 7 5 and 37°C for 60 minutes Rabbits injected with 1 5 ml of incubation mixture.

so that the balance between insulinase and insulinase-inhibitor is in the direction of an augmentation in the insulinase activity of slices. Accordingly, it was postulated that an insulinase-inhibitor may be present in the stock laboratory diet of the rat. Assays of trichloracetic acid extracts of all the components of the stock laboratory diet revealed the presence of insulinase-inhibitor activity in corn gluten only.

liver *in vivo* They injected insulin into the splenic vein and compared the hypoglycæmia with that resulting from the injection of insulin into the femoral vein They demonstrated that the insulin was less effective when injected into the splenic vein than when injected into the femoral vein

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DISCUSSION

Lukens: I presume that the statement that "there is no insulinase in plasma" includes the testing of the plasma of diabetics?

Mirsky I admit that statement referred only to normal patients. The plasma taken from patients with diabetes tested in this manner showed minimal activity. The plasma from patients with insulin resistance may show activity, as Marsh and his associates demonstrated. We are convinced that in the plasma of such patients there may be a system which does destroy insulin and which is not proteolytic

Lukens: Since you were testing this in the liver slice, have you

method we are using It is for such reasons that for the last two years

inactivation does or does not occur.

which indicates the presence of insulinase We have found it in slices, Levine's group have demonstrated the inactivation of insulin by the

carbohydrate metabolism by mobilizing the glucose reserves and distributing them to the tissues. Following injections of these hormones there may be a rise in the blood sugar level (Janes and Nelson, 1940), a dilation of the capillaries of the skin (Reynolds, 1941) and uterus (Williams, 1948), and a deposition of glycogen in the vaginal and uterine epithelia (Robertson, Maddux and Allen, 1930). There is also some evidence, discussed below, of increases in the uterine respiratory rate and of the ATP and creatine phosphate content.

Methods

The analysis of the relationship between œstrogens, carbohydrate metabolism, and mitosis was carried out with mouse ear epidermis *in vitro*, and the method used was that described by Bullough and Johnson (1951*a*) Fragments of ear were incubated in a phosphate-buffered saline medium with added glucose, and were contained in Warburg flasks with a gas phase of oxygen Each experiment, carried out at 38°C., was continued for five hours. During the first hour those mitoses which were already present when the ears were removed from the mice were allowed to pass to the telophase Colchicine was then tipped from the side-arm to arrest in the metaphase any mitoses which developed during the subsequent four hours. Finally the ear fragments were fixed and sectioned, and the numbers of mitoses present were counted.

One advantage of this technique was that no unknown factors were introduced with the medium, as would have been the case if serum or embryo extracts had been used. Unknown factors were, of course, introduced with the ear fragments themselves, and, as far as could be ascertained, the most important of these seemed to be the size of the carbohydrate reserves within the epidermal cells (although in a normal mouse these were found to be so low that they could be largely disregarded), and the hormonal complex present in the mouse tissues at the time of death. This second factor proved to be highly important, and because of the variations in the epidermal œstrogen content during the œstrous cycle, normal female

PART VI

SEX HORMONES, PREGNANCY AND CARBOHYDRATE METABOLISM

ŒSTROGENS, CARBOHYDRATE METABOLISM, AND MITOSIS

WILLIAM S BULLOUGH

Introduction

It has long been recognized that œstrogenic hormones stimulate the growth by mitosis of the female accessory sexual structures (Burrows, 1949), and more recently it has been appreciated that this mitogenic action of the œstrogens is also felt in a variety of non-sexual tissues (Bullough, 1946) In particular, the effects of these hormones on mitotic activity have been studied in mouse epidermis (Bullough, 1950*a*, *b*). Even more recently, a study of the physiology of epidermal cell division has led to the striking demonstration that by far the most critical single factor determining the degree of mitotic activity is the rate of epidermal energy production (Bullough, 1952). The Malpighian cells are only able to divide in aerobic conditions when significant quantities of energy are produced through the tricarboxylic acid cycle (Bullough and Johnson, 1951*b*).

Having thus demonstrated that two factors, the presence of œstrogenic hormones and a high rate of energy production, both stimulate epidermal mitosis, the question naturally arises whether all, or part, of the mitogenic action of these hormones is obtained through a stimulus to energy production. Already it is known that œstrogens do in some way affect

to avoid it by substituting L-lactate as substrate in place of glucose. In a preliminary experiment the optimum concentration of L-lactate was determined, and it was found to approximate to 0·02M as in the case of glucose. An experiment was then carried out with œstrone, and the results are summarized in Table II.

Table II

[illegible] Unit [illegible] bated [illegible] Each

Substrate				
None	*0·02M glucose*		*0·02M L-lactate*	
	alone	*+10γ œstrone*	*alone*	*+10γ œstrone*
0·4 ± 0·06	6·5 ± 0·40	11·2 ± 0·81	13·8 ± 0·93	13·2 ± 0·67

Two important conclusions emerge from these figures. First, it is evident that optimum L-lactate is approximately twice as effective as a substrate for epidermal mitosis as is optimum glucose, and indeed the result obtained with L-lactate is approximately the same as that obtained with glucose plus œstrone. The second conclusion is that when œstrone is added to L-lactate no further stimulus is obtained. Similar mitosis counts were later made when pyruvate was used as substrate in place of L-lactate, and all these results are clearly in agreement with the suggestion that the point of action of œstrone is at some stage of carbohydrate metabolism between extracellular glucose and intra-cellular pyruvate.

Œstrone and Hexokinase

When enquiring which of the pre-pyruvate stages may be the one primarily affected by œstrogenic hormones, consideration was given to the fact that, on the one hand, epidermal cells show little or no sign of glycogen deposition, while, on the

mice could not be used. While ovariectomized mice were tried with success, it was found much simpler to use normal adult males. When well-fed and kept in uniform conditions, it was found that male mice maintain a relatively constant hormonal state, and that their epidermal mitotic rate responds promptly to the presence of œstrone *in vitro*. The following experiments were therefore all based on the ear epidermis of normal Kreyberg's WLL male mice of between three and six months of age.

Œstrone and Glucolysis

Confirmation was first obtained that œstrogenic hormones, such as œstrone, are able to stimulate epidermal mitotic activity *in vitro* as readily as they do *in vivo*. A typical experiment is recorded in Table I and it can be seen that œstrone

Table I

AVERAGE NUMBERS OF MITOSES ARRESTED BY COLCHICINE IN 4 HR. IN UNIT LENGTHS (1 CM) OF SECTIONS 7μ THICK OF EAR EPIDERMIS INCUBATED AT 88°C IN SALINE MEDIUM WITH 0 02M GLUCOSE AND AN OXYGEN GAS PHASE EACH FIGURE IS THE AVERAGE OF 10 OBSERVATIONS

Œstrone concentration per 4 ml culture medium				
0	1γ	5γ	10γ	50γ
7 7 $\pm$ 0 41	8 2 $\pm$ 0 39	14 2 $\pm$ 1 67	15 0 $\pm$ 0 83	11 3 $\pm$ 0 45

in optimum concentration causes an approximate doubling of the mitotic rate.

A working hypothesis was then adopted that hormones such as œstrone may exert at least part of their mitogenic action through some stimulus to energy production within the epidermal cells, or, in other words, that they may facilitate some rate-limiting reaction in the process of energy production.

The first test of this hypothesis involved an attempt to locate the approximate position of the supposed rate-limiting reaction by discovering whether it is situated before or after the end of glucolysis. If it lies at some point between extracellular glucose and the end of glucolysis it should be possible

to avoid it by substituting L-lactate as substrate in place of glucose. In a preliminary experiment the optimum concentration of L-lactate was determined, and it was found to approximate to 0·02M as in the case of glucose. An experiment was then carried out with œstrone, and the results are summarized in Table II.

Table II

AVERAGE NUMBERS OF MITOSES ARRESTED BY COLCHICINE IN 4 HR IN UNIT LENGTHS (1 CM) OF SECTIONS 7μ THICK OF EAR EPIDERMIS INCUBATED AT 38°C IN 4 ML. SALINE MEDIUM WITH AN OXYGEN GAS PHASE EACH FIGURE IS THE AVERAGE OF 10 OBSERVATIONS

Substrate				
None	0·02M *glucose*		0·02M *L-lactate*	
	alone	+10γ *œstrone*	*alone*	+10γ *œstrone*
0·4± 0·06	6·5± 0·40	11·2± 0·81	13·8± 0·93	13·2± 0·67

Two important conclusions emerge from these figures. First, it is evident that optimum L-lactate is approximately twice as effective as a substrate for epidermal mitosis as is optimum glucose, and indeed the result obtained with L-lactate is approximately the same as that obtained with glucose plus œstrone. The second conclusion is that when œstrone is added to L-lactate no further stimulus is obtained. Similar mitosis counts were later made when pyruvate was used as substrate in place of L-lactate, and all these results are clearly in agreement with the suggestion that the point of action of œstrone is at some stage of carbohydrate metabolism between extracellular glucose and intra-cellular pyruvate

Œstrone and Hexokinase

When enquiring which of the pre-pyruvate stages may be the one primarily affected by œstrogenic hormones, consideration was given to the fact that, on the one hand, epidermal cells show little or no sign of glycogen deposition, while, on the

other, they have a low rate of energy production (QO_2= approximately 1·6 to 2·2). It appeared that if only one rate-limiting step exists and if this lies between glucose-6-phosphate and pyruvate then there should be no bar to active glycogen deposition, while, alternatively, if it lies between glucose-6-phosphate and glycogen then there should be no bar to active energy production. Such an argument suggested that the critical step might be the only one common to both these processes, namely the hexokinase reaction, and the effect of œstrone on this reaction was next examined.

If the mitogenic action of œstrone is primarily due to a facilitation of the hexokinase reaction, then it should clearly be possible to obtain the œstrogen effect by means of insulin. Preliminary experiments were performed, with glucose as substrate, to test this and to discover the optimum insulin concentration. After confirmation had been obtained that insulin *has a strong mitogenic action, the experiment recorded in* Table III was carried out.

Table III

AVERAGE NUMBERS OF MITOSES ARRESTED BY COLCHICINE IN 4 HR IN UNIT LENGTHS (1 CM) OF SECTIONS 7μ THICK OF EAR EPIDERMIS INCUBATED AT 38°C IN 4 ML. SALINE MEDIUM AND AN OXYGEN GAS PHASE EACH FIGURE IS THE AVERAGE OF 10 OBSERVATIONS

Substrate				
None	*0 02M glucose*			
	alone	*+10γ œstrone*	*+50γ insulin*	*+10γ œstrone +50γ insulin*
1 3± 0 25	7 6± 0 36	11 3± 0 50	12 0± 1 19	11 2± 0 45

Evidently the mitogenic actions of optimum concentrations of œstrone and insulin are approximately the same, and furthermore these actions are not additive

With this support for the present hypothesis, attempts were next made to bypass the hexokinase reaction by using phosphorylated compounds as substrates in place of glucose. The

first attempt was made with glucose-1-phosphate, but both the samples obtained failed to support any epidermal mitotic activity at all. Indeed, with concentrations of more than 0·03M the cells were seen to be damaged A second attempt was then made with fructose-1,6-diphosphate, but this too failed to support any mitotic activity. An analysis of the results suggested that these and similar phosphorylated substances may be unable to penetrate the cell walls, and this line of attack had to be abandoned

Two further attempts were then made to avoid the hexokinase reaction The first was a development of the point made above, that in certain circumstances the carbohydrate reserves already in the epidermis at the time when it is separated from the mouse may affect the results subsequently obtained *in vitro*. An attempt was made to increase substantially these intracellular reserves on the theory that, since they must already have passed the hexokinase reaction, any œstrone added *in vitro* should be without effect on their rate of utilization The mice were given preliminary injections of from 10–40 mg. of glucose one hour before they were killed, and fragments of their ears were then incubated both with and without substrate and with and without œstrone. The results are shown in Table IV.

Table IV

Substrate	*Previous treatment of mice*			
	None	*injected 10 mg glucose*	*injected 20 mg glucose*	*injected 40 mg glucose*
none	1 8±0 16	5 3±0 50	11 2±0 65	12 1±0 58
none + 10γ œstrone	1 5±0 21	5 6±0 36	10 5±0 55	12 0±0 84
0 02M glucose	6 8±0 21	7 4±0 32	10 8±0 62	12 3±1 18
0 02M glucose + 10γ œstrone.	10 0±0 71	9 6±1 00	10 6±0 41	12 4±1 02

Evidently such preliminary treatment with glucose can raise considerably the mitotic rate *in vitro,* and it is clear that when the carbohydrate reserves are increased in this way the mitogenic action of œstrone is no longer seen.

It could, of course, be argued that one effect of such preliminary glucose injections would be an increased rate of insulin secretion, so that when the ear fragments were detached from the body they would contain an unusually large concentration of this hormone. However, it would seem that, while this factor might have influenced the glucose uptake, and therefore the mitotic rate, in those groups where glucose was supplied in the culture medium, it would be unlikely to have influenced the results obtained with a saline medium alone. These experiments may therefore be held to support the argument that the action of œstrone is on some early step in carbohydrate metabolism such as the hexokinase reaction.

Fructose and Epidermal Mitosis

In a final attempt to relate the œstrogenic hormones to some particular reaction in the early stages of carbohydrate metabolism, the value of fructose as a substrate was investigated. In introductory experiments, the reactions of the epidermis to fructose were examined in order to discover whether the tissue is capable of utilizing this sugar at all. The unexpected result was that fructose in optimum concentration (0·02M) is almost twice as effective in supporting epidermal mitosis as is glucose in optimum concentration.

The second question to be answered was whether the epidermis, like the liver (Cori, 1950), contains separate gluco- and fructo-kinases which provide separate routes of entry for glucose and fructose. Experiments were carried out with insulin and with growth hormone. It was found that, whereas with a substrate of glucose insulin stimulated and growth hormone inhibited mitotic activity, with a substrate of fructose both these hormones were without effect. This result seems to indicate clearly the existence of a glucokinase which

is influenced by these hormones and of a fructokinase which is not (see Table V).

Table V

AVERAGE NUMBERS OF MITOSES ARRESTED BY COLCHICINE IN 4 HR IN UNIT LENGTHS (1 CM.) OF SECTIONS 7μ THICK OF EAR EPIDERMIS INCUBATED AT 38°C IN 4 ML. SALINE MEDIUM AND AN OXYGEN GAS PHASE EACH FIGURE IS THE AVERAGE OF 5 OBSERVATIONS

Substrate						
None	*0 02M glucose*			*0 02M fructose*		
	alone	*+50γ insulin*	*+500γ growth hormone*	*alone*	*+50γ insulin*	*+500γ growth hormone*
1 1± 0 33	7 7± 0 52	12 1± 0 89	1 9± 0 39	13 3± 0 72	11 9± 1 13	12 5± 0 36

Table VI

[illegible]

Substrate				
None	*0 02M glucose*		*0 02M fructose*	
	alone	*+10γ œstrone*	*alone*	*+10γ œstrone*
1 3± 0 25	7 6± 0 36	11 3± 0 50	11 9± 1 00	11 3± 0 67

Experiments were then performed with œstrone, and it was found that the results obtained with fructose as substrate compared closely with those previously obtained with L-lactate. It was confirmed that fructose, like L-lactate, is almost twice as effective as glucose in supporting epidermal mitotic activity, that the numbers of mitoses obtained with fructose are approximately equal to those obtained with glucose plus œstrone; and that when œstrone is added to fructose it fails to produce any mitogenic action

Thus the curious conclusions emerge first, that an efficient mechanism exists within the epidermis for the phosphorylation of fructose, a sugar which in normal circumstances can hardly ever reach the skin in significant quantities, and second that while the rate of epidermal glucose uptake is affected by the hormonal complex, that of epidermal fructose uptake is evidently not. More important for the present argument, however, is the evidence which the use of the fructokinase route is able to provide in support of the theory that one important action of œstrone, like that of insulin, is on the glucokinase reaction. There now seems to be little doubt that the greater part, if not the whole of the mitogenic action of œstrone is related to a facilitation of this reaction. The only other reaction which might be involved is the first step in glucolysis, the conversion of glucose-6-phosphate to fructofuranose-6-phosphate. However, with glucose as substrate, any stimulus to this reaction alone could obviously not cause glycogen deposition, and, as discussed below, such deposition is one of the characteristic effects of œstrogenic action

At this point several problems immediately present themselves. It is of obvious interest to try to discover how far an œstrogen (or other related compound) may act as a substitute for insulin, whether the actions of these two hormones are normally synergistic, or whether their effects *in vivo* are in fact quite separate. Then there is the still unexplored problem of the androgens and other steroid hormones, and the highly important question whether the reactions which can be demonstrated *in vitro* are also evident *in vivo*. These and other similar questions are now being studied

Conclusions

The main conclusion arising from the above data is that the mitogenic action of the œstrogenic hormones, at least in the epidermis, appears to depend to a high degree on some stimulus to the phosphorylation of glucose. It is thought that epidermis is normally half-starved for glucose (Bullough, 1952), and this may be taken as an indication either of its poor

blood supply or of its small capacity for absorbing such glucose as is available in its vicinity. Thus, at the moment it is impossible to suggest whether the œstrogenic hormones may act by stimulating the phosphorylation of that glucose which has already penetrated into the cell, or whether they may assist glucose uptake by stimulating its phosphorylation at the cell surface.

If the œstrogens indeed facilitate the glucokinase reaction, it may be expected that they will stimulate both energy production and glycogen deposition Neither process is easy to demonstrate in the epidermis. Even in optimum conditions the rate of glucose intake is apparently low: the QO_2 remains at about 2 0, and glycogen cannot usually be seen in the cells However, in more active tissues, such as those of the uterus, the œstrogens are reported to have a powerful effect in stimulating both anaerobic glycolysis and respiration (Kerly, 1940, Carroll, 1942), and in increasing the ATP and creatine phosphate concentrations (Menkes and Csapo, 1952). Similarly, it is well known that the œstrogenic hormones induce such heavy deposits of glycogen in the cells of the lining epithelia of parts of the Mullerian duct that vaginal smear techniques based on the staining of this glycogen have been devised (Burrows, 1949).

Since stress has been laid on the similarities of action of œstrone and insulin, it is important to preserve a proper balance by indicating their differences Clearly these differences must be considerable or the œstrogens would have been found to be fully effective against diabetes, while insulin would have been recognized to be an œstrogenic hormone. While the details of the differences have not yet been properly explored, one point of possible significance has already emerged, namely that, while insulin has an effect in a tissue mince (Krebs and Eggleston, 1938), the œstrogenic hormones apparently have not In complete contrast to the present argument, œstradiol has been found to induce a respiratory inhibition in liver homogenate (Guidry, Segaloff and Altschul, 1952). While such a contradictory result may prove to be

explicable in terms of dosage, it seems equally possible that the route through which œstrone influences the glucokinase reaction is normally destroyed by mincing.

Evidence is now accumulating that the glucokinase reaction may be particularly susceptible to hormonal interference. There are two hormones known, the glucocorticoid hormone and the pituitary growth hormone, which inhibit this reaction, and two others, insulin and œstrogenic hormone, which stimulate it. Cori (1950) has suggested the possibility that the two inhibitors may normally act synergistically, and the possibility must not be overlooked that some similar situation may exist in the case of insulin and œstrogen.

In several respects the present paper is a postscript to that of Houssay (1951), and it is interesting to reconsider briefly the views there expressed on the relation between the gonad hormones and experimental diabetes. Abundant evidence was put forward to show that the presence of active ovaries is a protection against diabetes in rats from which 95 per cent of the pancreas has been removed. This protection is largely destroyed by ovariectomy, but it can be replaced by injections of œstrogenic hormones. In analysing the antidiabetogenic action of these hormones he remarked that "the peripheral action of œstrogens upon carbohydrate metabolism is not yet well known," and he suggested that their protective value "seems to be due chiefly to the fact that they produce hypertrophy and hyperplasia of the islets of Langerhans in the pancreas " The mitogenic action of œstrogenic hormones on the cells of the islets of Langerhans has also been described by Bullough (1946), and evidence reviewed by Burrows (1949) indicates that these hormones may stimulate, directly or indirectly, the rate of insulin secretion.

It therefore appears that, while *in vitro* the action of œstrone on the epidermis may be primarily through a stimulation of the glucokinase reaction, *in vivo* its action may be much more complex, affecting the glucokinase reaction, the rate of cell multiplication in the islets of Langerhans, and the rate of insulin secretion.

Summary

1. The relation between the œstrogenic hormones and carbohydrate metabolism has been explored, using the rate of mitosis in mouse ear epidermis as indicator. The experiments were carried out in a phosphate-buffered saline medium and an oxygen gas phase.

2. With glucose as substrate œstrone induces a doubling of the mitotic rate, but with L-lactate or pyruvate as substrate the double rate is obtained without the addition of œstrone, which indeed provides no further stimulus.

3. The action of œstrone thus appears to be on some rate-limiting reaction between the extra-cellular glucose and intra-cellular pyruvate, and experiments have been performed which indicate that the hexokinase reaction is the one most probably involved

4. It has been found that mouse ear epidermis contains separate gluco- and fructo-kinases. With fructose, as with L-lactate, the mitotic rate is double that obtained with glucose, and the addition of œstrone has no effect. This is taken to indicate that the reaction facilitated by œstrone is either glucose ⟶ glucose-6-phosphate or glucose-6-phosphate ⟶ fructofuranose-6-phosphate, and arguments are advanced in favour of the first alternative.

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DISCUSSION

BEST Can you see all the cells here—how do you count them?

BULLOUGH: After fixation, fragments of ear are sectioned at 7μ, and the numbers of mitoses are then counted in 1 cm. lengths of the cut epidermis I suppose that strictly one ought to measure mitotic activity as, say, the numbers of mitoses/10,000 cells, but in fact the labour involved would be enormous and I have found the slight approximation involved in my method to be unimportant So those figures (1,7,14) which I put on the board are actually 1,7,14 mitoses/cm. length of ear epidermis cut 7μ thick

BEST You mentioned that there was no glycogen present Do you get glycogen formed in the epidermis?

BULLOUGH It forms shortly after wounding, and also after irritation with such a substance as croton oil. Whenever there is inflammation I think

BEST.

BULL

YOUN

will be to try to discover how these hormones interact when they are both present together

YOUNG: Is it necessary for us to assume that they both have an action in the hexokinase system, even though they do antagonize each other? It seems to me that the amount of ATP available at the cell surface might be a limiting factor, and that under the influence of

may

isulin.

ystem

it the

cell surface.

BULLOUGH Yes, that is quite possible. It is an open question whether the hormonal action takes place inside the cell or on the cell surface. However, we do know that the carbohydrate content of the epidermis is very much lower than that of the blood, and that therefore the gradient is in a normal direction, glucose entering with the gradient and

not against it This entry may be a process of simple diffusion and the hormones may act inside the cell, or alternatively entry may be an [illegible]

come limiting as long as glycolysis is going on.

Young· The amount of ATP available at the cell surface may not necessarily be indicated by the total ATP.

Best: These blocks seem to go either in one step or two steps, was that just the way you made your observations?

Bullough: No, that is the way it works out, I don't know why With insulin or œstrone you can get just about double the normal mitotic rate. Incidentally, you can also obtain this doubling without œstrone in the culture medium if the hormone is injected into the animal before the ear is taken off. Œstrone does not wash out of the ear fragments

Lawrence· It may be of interest in relation to œstrogen action and insulin that clinically speaking it seems to me quite certain that for a day or two before the menstrual period, women patients have a great tendency to develop hypoglycæmia, in other words, the insulin seems to be acting more strongly one or two days before the first day of the period, after which the usual balance returns

Dickens· Have you tried any what I call "near œstrogens," i e. substances which are not œstrogenic but which have closely related structures? The reason I ask this is because there was a paper by McShan and Meyer, of Madison, in which they said that œstrogens specifically inhibited succinoxidase Case and I re-investigated that and it seems that the inhibition is rather non-specifically connected with [illegible]

ship does not also play a part in this series.

[illegible]

have been using are chemically only remotely related to stilbœstrol.

[illegible]

[illegible] are nonœstrogenic would also do it

Bullough I'm hoping to test one or two of these near-related

non-œstrogenic substances this summer, but the answer to your question may take a little time to work out.

DICKENS About the fructose utilization, there is of course the well known observation that brain cortex (Loebel, *Biochem Z.*, 1925, 161, 219) oxidizes fructose without converting it to lactic acid: it has not the ability to form lactic acid. Greville and I (*Biochem. J.*, 1933, 27, 832) showed the same with the retina.

GAARENSTROOM May I ask Dr Bullough if he knows whether his effect with glucose, with lactate, and with fructose, is the maximal effect which can be obtained?

BULLOUGH That is an important question, and one about which I am rather worried. You will notice that in all the cases I have quoted we bumped up against this figure 14 and failed to get any higher I am a little suspicious about this Certainly, *in vivo*, this is not the maximal

of the experiments *In vivo* the maximum reaction is seen after about 24 hours; *in vitro* no experiment has continued longer than 5 hours.

GAARENSTROOM Did you ever use lower doses of fructose or lactate?

BULLOUGH Yes, but these were the optimum doses

NEEDHAM: May I ask if it is possible to get enough material to do enzyme experiments? Also if one could do enzyme experiments, how would they reflect what is happening in the epidermis in relation to what is happening in the rest of the skin? Is it possible to separate the epidermis from the rest of the skin?

BULLOUGH. Yes, you can separate epidermis quite readily from

snag there would be to get rid of the hairs. Of course the hairs themselves are inert, but the hair follicles are highly active in a cyclical

Possibly, however, you could
you suggest,
happy about
is, one would
han in males

ing point. It is clear
creasing energy pro-
ses which depend on
the epidermis The
he mitotic rate, you
tion and with it the
ide limits, however
high you raise the do not cause much

phenomenon

SWYER This is a very nice explanation, but it raises still further anomalies. Does it mean that females slough their skins more rapidly than males, and that females secrete more sebaceous material? I thought it was the other way round

BULLOUGH You may possibly be right, much observation remains to be made before a complete answer can be given

WOLSTENHOLME In conditions like acne which you mainly get in adolescence, often worse at menstrual periods, you may use œstrogens in order to get that sloughing and to stop the sebaceous secretion; you also may treat your acne with androgens or adrenocortical extracts.

BULLOUGH It's very curious that it has been reported that if you

sebum

It seems that continuous treatment with œstrogens *in vivo* causes many complications For instance the increased mitotic activity lasts for less than 48 hours, and then even though you continue œstrogen treatment, the mitotic activity cuts right down. Now the only way I

pituitary hormones, and not to the œstrogens themselves

LONG Some years ago we noticed what has been called the cortico-

BULLOUGH Yes, you can also prevent the mitotic cut-out if you take out the pituitary instead of the adrenals, either will do. The route seems to be through the pituitary, down to the adrenals

LONG. Levin showed that long-continued administration of these

substances produced very large adrenal glands in his rats They were practically depleted of lipid too.

BEST: There is a large increase in the weight of the islands of Langerhans after prolonged administration of œstrogens. Dr. R. E Haist in my department has been studying this for some years. You get it with stilbœstrol and with progesterone.

HORMONAL ASPECTS OF CARBOHYDRATE METABOLISM IN SEMEN AND MALE REPRODUCTIVE ORGANS

T. MANN

THE composition of semen is subject to considerable variation according to the relative proportion of its chief *components, the spermatozoa and the seminal plasma.* The seminal plasma, which is a mixture of secretions produced by the male accessory glands of reproduction, depends, so far as volume and composition are concerned, upon the variable contribution of these organs. The output of the accessory glands is governed by the actual size and storage capacity of each of the glands, particularly the epididymides, prostate, seminal vesicles and Cowper's glands. These are the facts which underlie the striking fluctuations encountered in the make-up of semen from different species and also from different individuals belonging to the same species These differences apply equally to morphological as well as to chemical characteristics of semen, as can be seen from Table I In all five species listed in this table, the chief carbohydrate constituent of the semen is fructose (Mann, 1946) This sugar

Table I

SPECIES DIFFERENCE IN COMPOSITION OF SEMEN

	Man	*Bull*	*Ram*	*Rabbit*	*Boar*
Volume of ejaculate (ml)	2–6	2–8	0 7–2	0 4–1	150–500
Sperm density (thousand/μl)	50–150	300–2000	2000–5000	100–2000	25–300
Fructose (mg /100 ml)	50–500	150–950	200–500	500–330	5–50
Citric acid (mg /100 ml)	80–800	300–1000	80–600	50–1200	30–300

does not occur in the sperm cells as such but forms part of the seminal plasma, and is derived from the seminal vesicles or functionally related glandular structures. At the site of their origin in the testes, the spermatozoa, immotile, have no fructose at their disposal. They come into contact with fructose during the ejaculation, and it is only then that they acquire the substrate for the energy-yielding process of sperm fructolysis.

Less pronounced but nevertheless highly significant, are the individual variations which occur in the composition of secretory fluids obtained directly from the accessory glands of reproduction. This is illustrated by Table II which gives the results of some recent chemical analyses carried out on

Table II

INDIVIDUAL DIFFERENCES IN COMPOSITION OF BOAR VESICULAR SECRETION

Boar (no)	Total volume of secretion (ml)	Fructose (mg /100 ml)	citric acid (mg /100 ml)	Ergothioneine (mg /100 ml)	Inositol (mg /100 ml)
1	30	15	250	82	2335
2	52	23	139	95	2590
3	170	55	295	84	2640
4	360	108	238	106	2350
5	550	124	981	89	2135

the seminal vesicle secretion of several boars, and includes, apart from fructose and citric acid, two newly discovered constituents of the boar vesicular secretion, that is ergothioneine (Leone and Mann, 1951) and meso-inositol (Mann, 1951*a*). It will be noted that the level of fructose and citric acid fluctuates much more than that of ergothioneine or inositol; furthermore a distinct parallelism can be observed between the volume of the secretion and the concentration of fructose, but not of ergothioneine.

Metabolic Effects of the Male Sex Hormone

Among the factors which control the size and the secretory capacity of the male accessory organs, and thus determine

the actual volume and composition of the seminal plasma, the testicular hormone ranks highest in importance. In the past, it was usual to assess the relationship between the activity of the male sex hormone and the functional state of the male accessory organs on purely morphological grounds Thus, for example, in order to define the response of rats to castration and to the action of testosterone, the changes in weight of say, the prostate or seminal vesicles, were taken as a measure. However, such an assay can be performed on the organs of the animal once only, that is after it has been killed. Therefore, the practical value of this procedure, particularly as applied to endocrinological studies in man or the higher animals, is obviously limited. It occurred to us that it should be possible to obviate these difficulties if we could prove that the activity of the testicular hormone in the male is reflected in the chemical composition of the seminal plasma, because then we could substitute the morphological criteria with accurate chemical measurements in ejaculated semen, and elaborate "hormone indicator tests" on a quantitative chemical basis.

The first experiments in this direction, carried out on rabbits, showed that within two or three weeks after castration, the content of fructose in the rabbit seminal plasma declined from 500 μg. to less than 20 μg. per ejaculate, and that the post-castration fall could be prevented or, if already developed, restored by the subcutaneous implantation of testosterone (Mann and Parsons, 1947) The sensitivity and adaptability of the "fructose test" can best be judged by the following experiment for which six bull calves were used (Mann, Davies and Humphrey, 1949). These animals were castrated when two to three weeks old, which is several months before the appearance of either fructose or citric acid in the seminal glands Seven months later, two of the castrated animals were implanted subcutaneously with 500 mg. testosterone each, and, together with the four untreated control animals, they were kept for another four weeks. All six animals were then slaughtered, and the seminal glands analysed. Histologically, the difference

between the seminal glands of the testosterone-treated and untreated animals was hardly noticeable; the tubules were approximately of the same diameter, their epithelium of about the same height. Chemical analysis on the other hand of fructose and citric acid most convincingly showed that whereas the seminal glands of the hormone-untreated calves had no more than a trace of fructose and no citric acid, those of the two testosterone-treated animals contained 48 and 55 mg. per cent fructose, and 23 and 16 mg. per cent citric acid respectively.

Here are some of the problems in sex endocrinology which were investigated during the last four years with the aid of either the "fructose test" or the "citric acid test" as indicators of male sex hormone activity.

Time Relationship between the Spermatogenesis and the Onset of Secretory Activity in the Male Accessory Organs. In young normally developing males, fructose and citric acid appear in the accessory glands of reproduction at an early age, well in advance of any signs of active spermatogenesis (Davies and Mann, 1947) In bull-calves, for example, the secretion of both fructose and citric acid is well under way in animals which are no more than four months old, whereas the first mature spermatozoa appear in the bull at the age of about twelve months (Mann, Davies and Humphrey, 1949) Since the secretion of fructose and citric acid has been shown to depend on the presence of the male sex hormone, it must be concluded that this hormone begins to function in the male body well in advance of the actual spermatogenesis.

Quantitative Assay of Testosterone. There is a proportional relationship between the dose of testosterone injected into castrated rats, and the response of the accessory organs expressed by their ability to produce fructose and citric acid (Mann and Parsons, 1950), this made it possible to use the quantitative determinations of fructose and citric acid as a sensitive method for assaying small amounts of testosterone.

Androgenic Potency of Progesterone. When castrated male rats were injected with 25 mg. progesterone daily, for three

weeks, the increase in size of the accessory glands was insignificant. Chemically, however, a distinct effect was detected: from the values for fructose and citric acid we were able to establish that in the rat, the androgenic potency of 25 mg. progesterone was just above that of 0·005 mg. testosterone propionate (Price, Mann and Lutwak-Mann, 1949).

Hormone-induced Formation of Fructose and Citric Acid in Transplants from Male Accessory Glands of Reproduction. The ability of the male accessory glands to elaborate fructose and citric acid does not necessarily depend upon the preservation of intact anatomical links with the rest of the male reproductive tract. Small fragments of rat accessory gland tissue, 1 mg. in weight, transplanted subcutaneously into adult male hosts, grew well, and when dissected after two or three months of subcutaneous development, showed a high content of fructose and citric acid. Such transplants could be grown equally successfully in female hosts, provided, however, that these animals were maintained under continuous treatment with testosterone propionate (Lutwak-Mann, Mann and Price, 1949)

Rôle of Hypophysis

In a discussion of the metabolic effects of the male sex hormone it is essential to bear in mind that in the living animal the output of the testicular hormone is determined by the activity of the anterior pituitary gland; that is why hypophysectomy is as effective as gonadectomy in abolishing the formation of the seminal fructose (Mann and Parsons, 1950) The dependence of the secretory function of the male accessory organs upon the pituitary gland activity is best illustrated by some studies on nutritionally deficient animals

Defective nutrition has been known for some time to have a detrimental influence upon the male reproductive tract, but earlier observers paid more attention to spermatogenesis than to the accessory organs. Recent studies showed, however, that one of the earliest symptoms which follow a reduced

calorie intake or certain other forms of malnutrition, particularly deficiency in vitamin B, is a drastic reduction in the secretory function of the male accessory organs (Lutwak-Mann and Mann, 1950, 1951). Three weeks of a vitamin B_1-deficient diet reduced the content of fructose and citric acid in rat coagulating glands and seminal vesicles to a practically post-castration level. However, the secretion of both fructose and citric acid in the rat accessory organs could be efficiently restored by *treatment with chorionic gonadotrophin*. Thus, the primary lesion due to inadequate food consumption is in the pituitary gland, and it is because of the pituitary hypofunction that the formation of seminal plasma comes nearly to a standstill.

A similar effect of malnutrition has also been demonstrated in the bull (Mann and Walton, 1952). The restriction of the food intake to half the normal quantity for a few months led to a progressive fall in fructose and citric acid in bull semen. Three months of such defective nutrition reduced the level of fructose from 865 mg. per cent to 250 mg per cent and the level of citric acid from 1340 mg per cent to 700 mg. per cent, on the other hand, the sperm density and motility still appeared unimpaired. These results show how important it is to pay attention to the general state of nutrition in experimental animals during biochemical or physiological investigations of semen.

Mechanism of Fructose Formation and the Rôle of Insulin

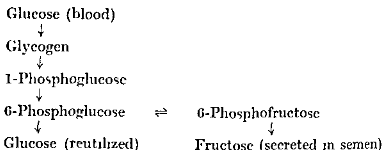
The first indication that glucose may be the precursor of seminal fructose was the outcome of *in vitro* experiments with bull seminal vesicle tissue, when it was shown that small amounts of fructose can be obtained from minced glandular tissue incubated with glucose (Mann and Lutwak-Mann, 1948). Subsequent investigations on rabbits disclosed the existence of a definite relationship between the blood glucose level and seminal fructose (Mann and Parsons, 1950). Experiments on

alloxan-diabetic rabbits have shown that soon after the rise of glucose in blood there was an unequivocal increase of fructose in semen; in severe alloxan diabetes (500 mg. per cent glucose in blood), the content of fructose in semen was found to exceed nearly five times the control values. When, however, in response to the administration of insulin the blood glucose level fell, the content of fructose in semen was also correspondingly reduced. Similar observations on the behaviour of seminal fructose were also made in human diabetes, semen from diabetic individuals was found to have an abnormally high level of fructose.

The findings on the relationship between blood glucose and seminal fructose led to enzymatic studies on the problem of fructose formation in accessory organs (Mann and Lutwak-Mann, 1951*a*, 1951*b*) Experiments *in vitro* have shown that extracts from bull seminal vesicles and from rat accessory glands liberate free sugars from phosphohexoses. 1-Phosphofructose is readily converted to free fructose and phosphate, and 6-phosphofructose into a mixture of fructose and glucose, through the combined action of isomerase and phosphatase When allowed to act on 1-phosphoglucose, these extracts produced from it a mixture of glycogen, 6-phosphoglucose, 6-phosphofructose, free glucose and free fructose, through the combined action of phosphorylase, phosphoglucomutase, isomerase and phosphatase.

The fact that out of a mixture of free glucose and free fructose, only one hexose, that is fructose, is present in semen under conditions *in vivo* may be due to a preferential re-utilization of glucose by the glandular tissue itself This interpretation is based on our earlier observations concerning the metabolism of tissue slices from rat coagulating glands which anaerobically were capable of glycolysing glucose more effectively than fructose (Mann and Lutwak-Mann, 1948).

The following reactions based on information available up to date from *in vitro* and *in vivo* experiments, may explain the accumulation of fructose in the male accessory organs.



The fructose-generating accessory glands differ markedly in their carbohydrate metabolism from the spermatozoa which are endowed with the ability to utilize fructose anaerobically; the form of fructose utilized is probably fructofuranose. Apart from fructose, spermatozoa can also utilize glucose and mannose owing to the presence of hexokinase. If washed spermatozoa are allowed to act on glucose and fructose mixed in a 1·1 proportion, the rate of fructose utilization is reduced by more than 50 per cent This "sparing effect" of glucose on the utilization of fructose by spermatozoa is due to the competitive inhibition of sperm hexokinase (Mann, 1951*b*).

Concluding Remarks

As to the action of hormones upon the carbohydrate metabolism in the highly specialized tissues of the male reproductive tract, it would appear that so far as *insulin* is concerned, its effect on seminal fructose is not a direct one, but the outcome of changes brought about in the glucose level in blood The spectacular effects of the *male sex hormone* are undoubtedly also the result of a complicated sequence of processes which, however, occur in the male accessory organs themselves. The influence of the testicular hormone, while best demonstrated by the variations in the level of certain metabolites such as fructose or citric acid, is presumably primarily directed at the enzymic proteins concerned with intermediary reactions involved in the carbohydrate metabolism. However, even our present knowledge shows sufficiently clearly how careful and critical one must be in evaluating

the chemical findings in semen, particularly with regard to hormonal aspects. It is advisable before placing a final interpretation upon a change of say, fructose in semen, to take into consideration several possibilities, since the observed phenomenon may be due to a species characteristic, to individual fluctuations, to malnutrition, to a variation in the blood glucose level, to gonadotrophic effects, to a perfectly normal fluctuation in the activity level of the male sex hormone, to other androgens, possibly even some anti-androgenic substances, and finally, perhaps to some other factors of which we may not be aware at present

That the carbohydrate metabolism of the male reproductive organs is by no means a simple one, is best indicated by the occurrence, in addition to fructose, of a cyclitol closely related to it, namely meso-inositol (Mann, 1951*a*) Inositol is found in a particularly high concentration in boar semen, and it is produced in the seminal vesicle, that is in the same gland which also produces fructose. The relation of inositol to fructose, its biogenesis and its function in semen still remain to be elucidated

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DISCUSSION

C. F. Cori: Dr. Mann, you promised to say what ergothioneine is doing.

Mann Ergothioneine, i.e the betaine of thiolhistidine, which Dr Leone and I isolated in crystalline state from boar semen, is an SH-

Both motility and fructolysis are extinguished completely by the addition of 10^{-3} M- iodosobenzoic acid If, however, iodosobenzoic acid is added together with ergothioneine (in a physiological concentration), there is no inhibition of either motility or fructolysis The antagonistic action of ergothioneine is not confined to iodosobenzoate but applies to other reagents which affect SH groups in sperm. Of particular interest is the counteraction by ergothioneine of the hydrogen peroxide effect. Hydrogen peroxide is known to be produced under certain conditions by the spermatozoa themselves and unless removed

THE EFFECT OF CASTRATION AND STEROID THERAPY ON SEMINAL PLASMA WITH RESPECT TO FRUCTOSE UTILIZATION BY NORMAL BULL SPERM*

F. X. GASSNER, E. R RUTHERFORD, M L HOPWOOD and H J. HILL

THE demonstration by Mann (1946*a*) that fructose and not glucose is the glycolysable reducing sugar present in mammalian semen stimulated considerable interest in sperm physiology from a new and different angle. Seminal fructose is elaborated by the accessory male reproductive organs under the control of the testicular hormones and represents the principal nutrient substrate for the metabolic activity of spermatozoa. This was shown in various domestic animals by Mann (1946*b*), in rabbits by Davies and Mann (1947), Mann and Parsons (1947), and Parsons (1950), in rats and bulls by Mann, Lutwak-Mann and Price (1948), Mann, Davies and Humphrey (1949) These authors were able to show that fructose disappears from seminal plasma as well as from the accessory sex organs after castration and that implantation or injection of testosterone restores its normal levels.

Gassner, Hill and Sulzberger (1952) and Gassner (1952) confirmed and extended these findings with respect to the utilization of fructose by spermatozoa, the effects of castration and restitution with androgenic and œstrogenic steroids on seminal fructose of the bull, and the effects of gonadotrophins on the fructolysis index of sub-fertile breeder bulls.

While the restitution of the accessory sex organs of the

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Sum

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castrated bull is rapid and apparently complete relative to size and secretion of fructose and citric acid, the question arises. Is the seminal plasma of the hormone-stimulated castrate similar in chemical composition to that produced by the intact animal, and is it capable of supporting sperm metabolism as well as normal seminal plasma?

It is the purpose of this paper to report on the investigation of this problem.

Experimental

The procedure and results of a preliminary trial conducted and reported by Gassner (1952) were briefly as follows:—

A large sample of semen was obtained from a normal bull and separated into three aliquots. Two aliquots were separated into plasma and sperm by centrifugation. The sperm residues were repeatedly washed with buffer solution. One sample of reconstituted semen was obtained by combining one sperm residue with normal plasma in original proportions while another sample was obtained by combining the other sperm residue with plasma of high fructose content from a castrated bull. (This animal had been orchiectomized three months previously and was under treatment with pregnenolone and testosterone in an attempt to alleviate castration changes.) The results of castration and treatment with steroids of this bull are shown in Fig. 1. These sperm transfers were performed when the fructose content of the castrate plasma ranged from 600 to 800 mg. per cent. The normal semen aliquot and the two reconstituted semen samples were then examined for fructose content, sperm concentration and rate of fructolysis as outlined by Mann (1948)

Fig. 2 demonstrates that in six consecutive weekly trials the donor sperm were not able to utilize the fructose in castrate plasma to the same extent as was shown when they were reconstituted with normal plasma, since the rate of fructolysis was depressed by 38 to 65 per cent.

The question arose as to whether semen from different individuals may be intermixed without causing incompati-

bility which would interfere with normal metabolism of fructose by spermatozoa.

Semen samples from three normal bulls were analysed individually and in a mixture of equal proportions for sperm concentration, fructose content and rate of fructolysis The

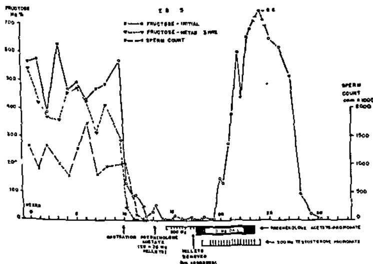


FIG 1 Effect of castration and steroid therapy on seminal fructose in the bull Sperm concentration, initial fructose content and fructose metabolized in three hours were normal before orchiectomy The rapid decline of seminal fructose after castration is evident Implantation or injection of pregnenolone acetate and propionate had no effect on the accessory sex glands since seminal fructose remained absent The addition of testosterone propionate (Perandren, Ciba) however, caused a rapid return of seminal fructose to well above the pre-castration level Upon withdrawal of the steroids fructose disappeared

mixed semen sample was separated into plasma and sperm fractions by centrifugation The plasma mixture and sperm mixture were then combined in original proportions and the fructolysis index was determined The results are tabulated in Table I and show that there is no interference with the

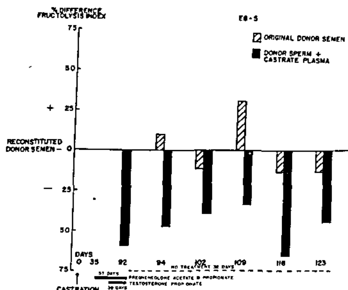


Fig. 2. *Effect of castration and steroid therapy on quality of seminal plasma*

Table I

Fructose Metabolism in Mixed Bull Semen

Semen sample	*Fructose* Init mg %		*Final* mg %	*Metab 3 hr* mg %		*Sperm ct* cmm × 1000		*Fructolysis index* 3 hr	
Bull 10	363		150	213		752		2 83	
Bull 13	385	Ave	18	367	Ave	1049	Ave	3 50	Ave
Bull 14	420	389	13	407	396	1457	1086	2 80	3 17
Whole semen mixture (Bulls 10, 13, 14)	373		13	360		1135		3 17	
Sperm mixture + Plasma mixture	369		6	363		1036		3 50	

rate of fructolysis when semen from different individuals is mixed. It is also demonstrated that the procedure employed in sperm transfer is accurate and that the results obtained are valid.

It appears from the foregoing that, since normal sperm was not able to utilize fully the fructose present in seminal plasma of castrates, the chemical composition of plasma from castrates restituted with steroids differs from the seminal plasma produced by the intact bull, either because one or more constituents are lacking or because of a shift in the quantitative relationship of seminal plasma components. These observations further suggest that the normal testicle may elaborate hormonal substances other than testosterone and œstrogen, or that the epididymal fluid may contribute nutrients which are not furnished by the accessory sex organ fluids and all of which are required by spermatozoa for the maintenance of normal fructose metabolism. In an attempt to shed further light on this interesting problem the following experiments were conducted:—

1. Effect of Castration and Maintenance with Testosterone on Seminal Plasma Quality

A normal bull was castrated and received, on the same day and every two days thereafter, 100 mg. of testosterone propionate (Perandren, Ciba) The effect on seminal fructose is shown in Fig. 3. The seminal fructose level remained high until a week after testosterone was withdrawn when a rapid loss of fructose occurred.

Normal bull sperm transferred at weekly intervals to plasma obtained from this castrate failed to utilize as much fructose as was shown to be the case when the sperm were combined with normal plasma (Fig 4).

2. Effect of Castration and Restitution with Testosterone on Seminal Plasma Quality

Another normal bull was castrated. No supporting steroid therapy was given in order to allow the accessory sex glands

to regress in size and function (Fig. 5). After seminal fructose dropped to near zero, 200 mg. of testosterone propionate (Perandren) were injected every two days. Seminal fructose returned rather rapidly and disappeared soon after the steroid was withdrawn. Following an eight week rest period testosterone thereapy was resumed with the result that the

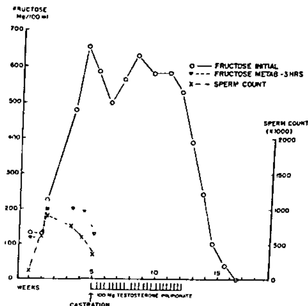


FIG 3 Seminal fructose in the bull—prevention of castration changes with testosterone.

seminal fructose again rose to the pre-castration level. Upon cessation of the therapy, fructose again disappeared.

Seminal plasma of this bull was tested repeatedly to determine its ability to support the utilization of fructose by normal sperm. The results are summarized in Fig. 6.

Two weeks after castration and before treatment with testosterone was begun, seminal fructose had declined to 27 mg. per cent. When 400 mg. per cent fructose were

added, only 20 per cent of the fructose present was metabolized by the donor sperm. Testosterone seemed to have an immediate effect on the seminal plasma since the rate of fructolysis was greater than that shown by the reconstituted donor semen Upon withdrawal of the steroid, only 30 per cent of the fructose present was utilized The effect of

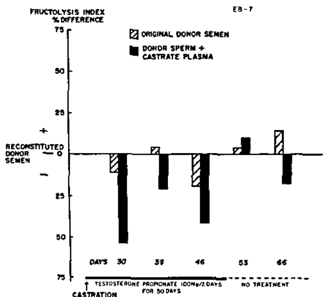


Fig 4 Effect of castration and testosterone therapy on quality of seminal plasma

resumption of steroid therapy is shown in that fructose was again more efficiently metabolized. Thereafter the seminal plasma quality seemed inadequate fully to support fructolysis by normal sperm. It should be pointed out that five months had elapsed since castration and that the response seen may well indicate a loss in target organ response, as observed by us in earlier experiments (Gassner *et al*, 1952).

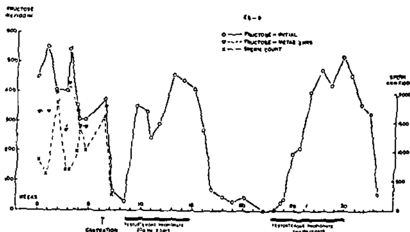


FIG. 5. Seminal fructose in the bull—effect of castration and restitution with testosterone.

FIG. 6. Effect of castration and testosterone therapy on quality of seminal plasma.

3. Effect of Vasectomy and Testosterone on the Quality of Seminal Plasma

Since in the vasectomized animal the testes remain *in situ*, the accessory sex glands remain under the trophic influence of the testis hormone and should produce a plasma normal

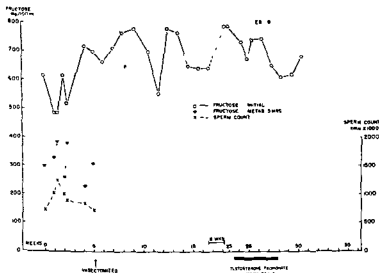


FIG. 7. Seminal fructose in the bull—effect of vasectomy and testosterone therapy

with respect to chemical constituents Therefore, it should behave in a similar fashion to normal seminal plasma deprived of sperm by centrifugation. To test this assumption vasoresection was performed on a normal bull The seminal plasma was analysed for fructose concentration at weekly intervals; Fig. 7 shows that the fructose content was in no way affected by vasectomy nor was it changed when testosterone propionate was given for a period of over four weeks. Normal bull sperm were transferred repeatedly to plasma obtained from this animal. The results shown in Fig. 8

indicate that while during the first two months after vasectomy the degree of fructose utilization by normal sperm gradually improved it remained below normal thereafter regardless of the fact that testosterone propionate was given.

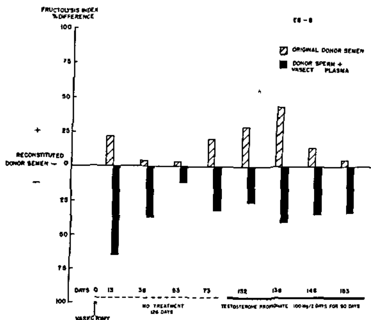


FIG. 8 Effect of vasectomy and testosterone therapy on quality of seminal plasma

Discussion

At the moment it is impossible to draw any definite conclusions from the results obtained. It appears that testosterone cannot fully compensate for castration changes brought about in the accessory sex organs, particularly as far as the chemical composition of their secretions is concerned. The response shown by the vasectomized animal is most puzzling. If our evaluation of the rôle of the governorship exerted by the testes over the accessory glands is correct, then there should be no difference in the metabolic behaviour

of normal sperm when reconstituted with either normal plasma or with plasma delivered by the vasectomized bull. A similar response to vasectomy was shown with respect to other seminal components, namely, the free amino acids We were able to show (Gassner and Hopwood, 1953) that the free amino acids disappear completely after castration as well as after vasectomy, but return, at least in part, when testosterone propionate is injected. It has been further shown by us that the amino acid pattern of fluids obtained from the ampullæ vasa deferentia as well as from the seminal vesicles of the normal bull is quite similar, although the ampullar fluid shows a higher concentration of amino acids per unit volume. This difference in concentration does not, however, account for the total lack of amino acids in the plasma of the vasectomized animal. This justifies the tentative conclusion that vasectomy affects the functional status of the accessory sex structures.

Another series of bulls, vasectomized or castrated and receiving androgens and œstrogens and mixtures thereof, are at the present time on trial Information received from those experiments have confirmed the observations reported here.

Summary

1. The method employed for the separation of normal bull semen into plasma and sperm with subsequent reconstitution appears to have no detrimental effect upon the ability of the sperm to metabolize fructose.

2. Similar results were obtained when semen specimens from three normal bulls were intermixed.

3 The immediate administration of testosterone propionate to a bull at the time of castration prevents the loss of seminal fructose resulting from castration. Seminal plasma from such an animal was not fully capable of maintaining the metabolism of fructose by normal sperm

4. Semen plasma of negligible fructose content from a bull, in which castration changes were allowed to develop before

testosterone therapy was begun, failed to support a normal rate of fructolysis since only 20 per cent of added fructose was utilized by normal sperm. Testosterone therapy caused a marked increase in both seminal fructose and its utilization. Subsequent withdrawal and resumption of steroid therapy produced similar results. Thereafter, in spite of continued treatment with testosterone, the seminal plasma of this castrate only partially supported fructose metabolism.

5. Vaso-resection in a bull had no adverse effect on either sex drive or the production of seminal fructose. However, when normal sperm was transferred to seminal plasma from this bull, the utilization of fructose was, contrary to expectations, markedly reduced. This would indicate that vasectomy adversely affects the functional status of the accessory sex organs.

The authors gratefully acknowledge the technical assistance of Lois Lucas. Thanks are due to F. E Houghton, Ciba Pharmaceutical Products, Inc , Summit, N.J., for the generous supply of Perandren (testosterone propionate)

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DISCUSSION

MANN: Normally fructolysis is correlated very strictly with sperm motility. By motility, I don't mean a phenomenon graded by microscopic observation as "one cross," "two crosses," etc., but a process

measured electrically by the new objective method of Rothschild You said, Dr. Gassner, that spermatozoa utilize fructose better in

fructose in the seminal plasma from normal and from castrated-hormone treated animals

SWYER: It seems that since vasectomy leads to the production of seminal plasma which is equally deficient, the epididymis might be contributing something in the intact animal

GASSNER Since the rate of fructolysis is closely correlated with motility and since normal sperm cells fail to utilize fructose fully in plasma from castrated and steroid-treated animals, Dr. Mann's inference with respect to retarded motility may be quite correct. We plan to employ Lord Rothschild's impedance test to answer this question.

We have considered also the problem of whether or not the epididymal fluid in the intact animal contributes substances to the seminal plasma which would be required for the utilization of fructose by spermatozoa While it was not difficult to obtain up to 10 ml. seminal vesicular fluid or about 1 ml from the ampullæ vasa deferentia, attempts to express epididymal fluid in quantities sufficient for analysis have failed Moreover, one has to be careful in manipulating the excised organs in order not to contaminate the secretions by tissue fluid from the non-glandular stroma The fluids expressed from the seminal vesicles and the ampullæ vasa deferentia of five normal bulls at slaughter were analysed for fructose, as well as for free amino-acids The concentration of these materials was remarkably uniform in all animals and was within the range found in seminal plasma It is possible that epididymal fluid furnishes substances of enzymatic nature, if so, probably in very small quantities Obviously, this question cannot be answered until

fructose by normal spermatozoa in a similar manner to what is seen when the castrates are used The present concept of the effect of vasectomy needs careful re-examination It has been assumed that vasectomy does not produce castration changes either in the accessory sex glands or in the pituitary. It is certainly true that in man vasectomy is performed regularly and years later the ejaculatory tract can be re-established resulting in normal reproductive function. We are continuing our investigation of this most puzzling problem

SOME ENDOCRINE STUDIES IN DIABETIC PREGNANCY

CHARLES H GRAY

THE most extensive studies of hormone excretion in normal pregnancy and in pregnancy complicated by toxæmia are those of Smith and Smith (1947). In a large proportion of cases of toxæmia of pregnancy they found a high gonadotrophin and low pregnandiol and œstrogen excretion which they interpreted as due to a diminished utilization of gonadotrophin in the placenta for the production of œstrogens and of progesterone. Their results led to the use of stilbœstrol and progesterone in the therapeutic and prophylactic treatment of toxæmic pregnancy. The Smiths included in their series a number of cases of diabetic pregnancy which many workers have assumed is associated with a toxæmia of pregnancy because of the high fœtal or neonatal mortality. White (1945) extended the studies of diabetic pregnancy and claimed to confirm the work of the Smiths both in respect of the laboratory studies as well as the efficacy of stilbœstrol and progesterone therapy Unfortunately the criteria of diabetes adopted by White are not clear and the fact that the patients in her series were kept in hospital throughout pregnancy throws confusion on the significance of her results of hormone treatment.

As part of a collaborative investigation with other laboratories, sponsored by the Medical Research Council and concerning certain aspects of diabetic pregnancy, we have determined pregnandiol and gonadotrophin excretion mostly at intervals of two weeks from about the twelfth week to term in fifty-one diabetic pregnancies. Pregnandiol was determined by the method of Sommerville, Marrian and Keller (1948) and chorionic gonadotrophin by the prostatic weight method of

Loraine (1950). Full statistical analyses of our results will ultimately be made with those obtained from other centres, but as far as they go Table I summarizes our results. In agreement with the results of Loraine and Matthew (1950) in pre-eclamptic toxæmia, high gonadotrophin excretion is found in about 45–55 per cent of cases whether pregnancy produces a surviving child or not, and pregnandiol excretion is only

Table I

CHORIONIC GONADOTROPHIN AND PREGNANDIOL EXCRETION IN DIABETIC PREGNANCY

Total number of cases = 51

	Pregnancy with surviving child		*Pregnancy with fœtal or neonatal death*	
Chorionic gonadotrophin				
High*	18	32	9	19
Normal	14		10	
Pregnandiol				
Normal†	30	32	17	19
Low	2		2	

*12,000 i u /24 hr before 30th week, 15,000 i.u /24 hr after 30th week
†45 mg /24 hr before end of pregnancy

rarely lower than normal It seemed obvious therefore that the relationship between unsuccessful pregnancy in diabetes and abnormal excretion of gonadotrophin in pregnancy was unlikely to be so direct as had been supposed by the Smiths or by White. The possibility of increased ACTH activity seemed not unattractive for it has long been recognized that the adrenals are enlarged in pregnancy (Ask-Upmark, 1926), and numerous workers have reported an increased excretion of adrenal cortical hormones in pregnancy (Devis, 1949; Parviainen, Soiva and Vartiainen, 1950, Tobian, 1949; Venning, 1946) Diabetes has also been shown to be associated with an increased excretion of formaldehydogenic steroids (McArthur, Sprague and Mason, 1950) and it seemed a possibility

that when pregnancy was complicated by diabetes, ACTH activity and adrenal cortical hormone excretion might be either qualitatively or quantitatively changed in those cases ending in a non-surviving child.

Dr. Parrott of King's College Hospital has determined the plasma ACTH in diabetic pregnancy but in view of criticisms which have been levelled at the significance of such measurements I do not propose presenting them in detail. As far as they go, it appears that there is no direct relationship between plasma ACTH and the outcome of diabetic pregnancy, but more cases need to be studied for it is not impossible that sudden changes in plasma concentration of this hormone may be of greater importance than the absolute level. Less direct means of assessing ACTH activity appeared to be necessary. Thus increased ACTH activity is usually associated with an increase in the urinary excretion of both 17-ketosteroids and especially the other adrenal cortical hormones and their metabolites.

Dr. Pond has examined cases of diabetic pregnancy with a micro-modification of the Dingemanse fractionation of 17-ketosteroids (Pond, 1951). The ketosteroids are separated by chromatography on alumina into eight separate fractions which include the non-alcoholic ketosteroids together with any chlorodehydroisoandrosterone, two fractions containing β-hydroxy-17-ketosteroids, fractions containing mainly androsterone and ætiocholanolone, two fractions containing 11-hydroxy-17-ketosteroids and a final one containing unidentified substances. Fig. 1 shows the pattern obtained with a normal female excreting a relatively large amount of total 17-ketosteroids, the pattern with a normal female excreting only 4 mg./day as well as that obtained from a female with Cushing's syndrome, who however excreted only 13 mg. total 17-ketosteroids per twenty-four hours. In this patient fractions 6 and 7 containing the 11-hydroxy-17-ketosteroids as well as fraction 5 containing ætiocholanolone were considerably increased above the normal range. On the other side of the figure there are the patterns obtained from three

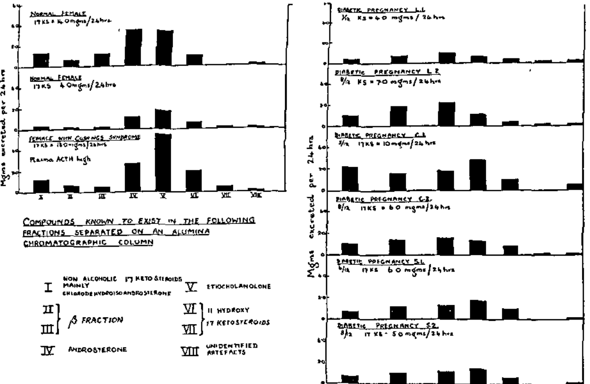


FIG 1. Pattern of 17 ketosteroid excretion in two normal females, in a Cushing's syndrome and in three pregnant diabetic patients.

cases of diabetic pregnancy, each of which was measured on two occasions. Only the third case had a living child. On the whole the total 17-ketosteroids tend to be lower than normal, but there is no increase in excretion of the 11-hydroxy-17-ketosteroids or alteration of the androsterone/ætiocholanolone ratio. The β-fraction appears to be increased, but 20-keto-steroids such as the pregnanolones are eluted in this fraction and although the Zimmermann reaction of these compounds is very weak the quantities excreted are so great that they have produced an apparent increase in the β-fraction

The problem of determination of adrenal cortical hormones and their metabolites in urine has not yet been solved for they are known to be excreted both as free steroids and as conjugated steroids, some of which are partially destroyed on acid hydrolysis while others require to be hydrolysed by β-glycuronidase (Cox and Marrian, 1951). None of the existing methods appeared suitable for our purpose and Miss de Courcy and Miss Lunnon have therefore examined four separate fractions by paper chromatography and determined the qualitative pattern of excretion and made a semi-quantitative assessment of the quantities. Fig. 2 shows the way in which these fractions were obtained The first fraction contained free steroids extractable with chloroform at neutral pH together with steroids readily split from their conjugates by acid and extractable with chloroform immediately after acidification. Our experience suggests that very little additional material is extractable by this acidification. The second fraction consisted of those extra steroids liberated as a result of hydrolysis in acid for 24 hours and this very rarely contained any detectable steroid. The third fraction consisted of those steroids liberated on enzymic hydrolysis, while the fourth consisted of chloroform soluble conjugates extractable with sodium carbonate during the purification of the first fraction Using the solvent systems described by Bush (1952) and Bush, de Courcy, Gray and Lunnon (1952), Compounds E and F, and tetrahydro-compound E have been identified with a fair degree of certainty. One spot has been tentatively

identified as corticosterone. Five unidentified spots, the ultra-violet fluorescence of which showed the presence of an unsaturated ketone group, have been provisionally labelled X_1, X_2, X_3, X_4 and X_5. Three unknown steroids with reducing activity but containing no $\alpha\beta$-unsaturated ketone group have been provisionally labelled R_3, R_6 and R_7. Fig 3 shows

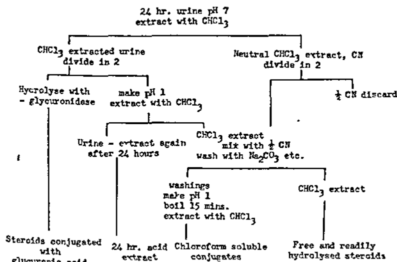


FIG. 2. Procedure for fractionation of urine for paper chromatography analysis of adrenal cortical hormones and their metabolites

the pattern of excretion of free and readily hydrolysed steroids excreted in ten normal females, eight normal pregnancies, seven diabetic pregnancies and three non-pregnant diabetics The assessment of the quantities of these compounds depended upon either the intensity of ultra-violet fluorescence or on the intensity of their reducing activity and is admittedly very approximate

The various compounds have been plotted in descending order of their polarity, i e. in the order of their R_f values. The vertical scale upon which the quantities of the various

compounds has been plotted has been drawn so that the highest block represents an excretion of over 500 μg./day. In our earlier experiments, no assessment of the intensity of the spots was made. In these cases, question marks in the diagram show that the substance was present but there is no record of how much.

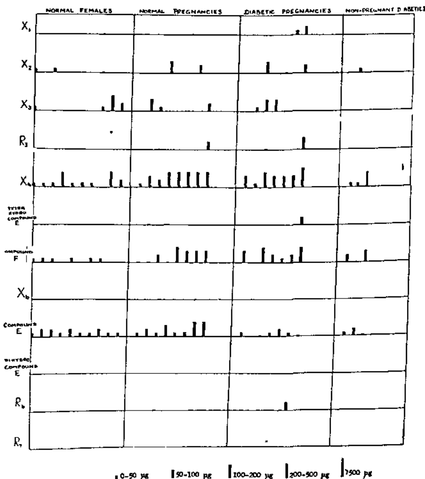


FIG. 3 Pattern of excretion of free and readily hydrolysed steroids

Free and Readily Hydrolysed Steroids

Non-pregnant Women

Compounds E and F are present each in amounts of 50–100 μg./day. About the same quantity of the αβ-unsaturated ketonic steroid, X_4, is also present. In the luteal phase of the menstrual cycle X_4 excretion appears to increase to about 250–500 μg./day.

Normal Pregnancy

Compounds E and F are present often, but not always, in increased amounts. X_4 is usually increased to amounts usually observed in the luteal phase in the non-pregnant. There is no obvious difference either quantitatively or qualitatively in pregnant diabetics.

Chloroform Soluble Conjugated Fractions

Fig 4 shows the pattern of excretion of chloroform soluble conjugated steroids. In non-pregnant females traces of Compounds E and F may sometimes, but not always, be present. X_4 was present in five of the ten cases and traces of corticosterone may also have been present No compound E appeared in this fraction in normal pregnancy and a trace was found in only two of the seven diabetic pregnancies. X_4 was present in small amounts in all eight normal pregnancies and in four of the seven diabetic pregnancies. Other compounds crop up irregularly but the quantities in this fraction are in general so small that no significance can be paid to any minor differences

Steroids Conjugated with Glucuronic Acid

Fig 5 describes the pattern of excretion of steroids conjugated with glucuronic acid and is particularly interesting. In non-pregnant women no compound F, X_1, X_2, or X_4 are present but occasionally traces of Compounds E, X_3, R_3 and R_7 may be recognized In all the subjects studied large

amounts of tetrohydro compound E were present while considerable quantities of other reducing steroids were usually, but not always, present. In most cases Compound X_8, present otherwise only in traces in the chloroform soluble conjugated

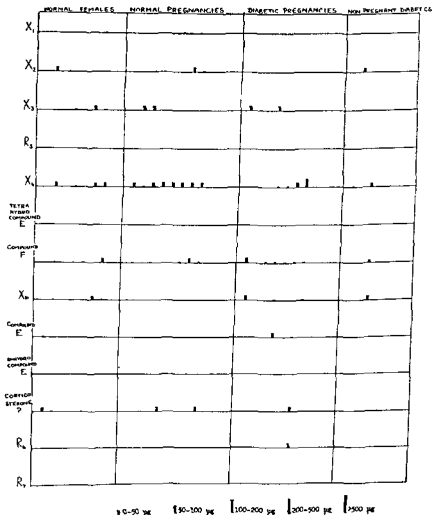


FIG. 4. Pattern of excretion of chloroform soluble conjugated steroids

fraction, is present especially during the luteal phase of the menstrual cycle when there are particularly large amounts. The hormones excreted in this fraction do not differ either qualitatively or, as far as could be judged, quantitatively from the normal in either normal or diabetic pregnancy.

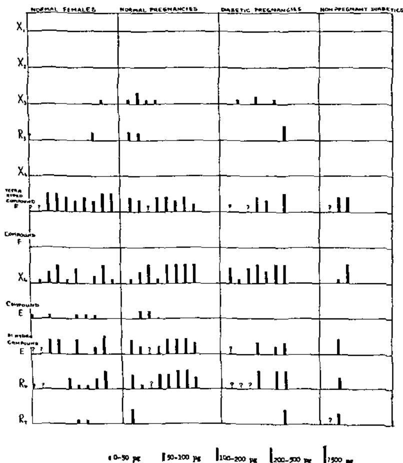


FIG 5 Pattern of excretion of steroids conjugated with glucuronic acid

amounts of tetrohydro compound E were present while considerable quantities of other reducing steroids were usually, but not always, present. In most cases Compound X_6, present otherwise only in traces in the chloroform soluble conjugated

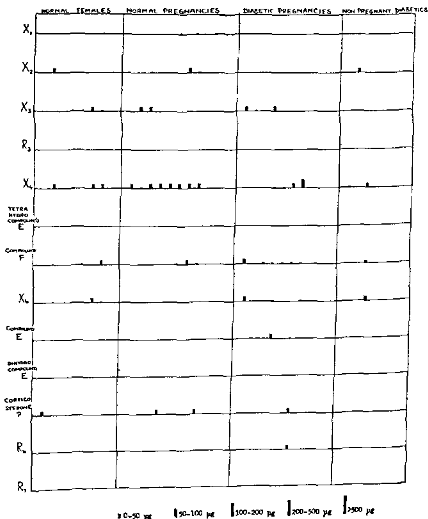


FIG. 4. Pattern of excretion of chloroform soluble conjugated steroids.

adrenal cortical hormones and their metabolites by Miss de Courcy and Miss Lunnon It is with their approval that I present a summary of results obtained.

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It would appear that *in pregnancy the same hormones and* their metabolites are present and together with other conjugates are excreted as in the normal person. The quantities *excreted are* usually greater, but during the luteal phase of the menstrual cycle non-pregnant women may on occasion exhibit as great an excretion as observed in pregnancy. In the cases we have examined so far there is no essential difference in the adrenal cortical hormone excretion in normal and in diabetic pregnancy.

Several points require some emphasis. We have not estimated pregnanolones nor œstrogens, nor the adrenal cortical hormone metabolite recently separated by Grundy, Simpson and Tait (1952) which is so very active in producing sodium retention. This last compound would not necessarily be detected in our experiments. Also we have not examined our chromatograms for steroids containing neither an $\alpha\beta$-unsaturated ketonic group nor a reducing side chain As far as *our studies go it appears that the relationship between abnormal* hormone excretion and the outcome of diabetic pregnancy must be incidental and that there is little reason to believe *any causal relationship between the two. It is important to* emphasize, however, that the complications of diabetic pregnancy are not identical with pre-eclamptic toxæmia. We have in no way disproved the work of Smith and Smith in relation to this last condition although it is important to realize that these workers' cases were heavily weighted with patients with diabetic pregnancy. The complications of diabetic pregnancy are clinically quite different from the syndrome of pre-eclamptic toxæmia and much confusion has arisen in the past by workers assuming that the ætiology of the two conditions are necessarily identical

The work presented represents the results of a long term investigation of diabetic pregnancy carried out by a team of workers at King's College Hospital. The gonadotrophin and pregnandiol determinations were performed by Miss Wood, Miss Edwards and Mrs. Gill, plasma ACTH was determined by Dr. Parrott, ketosteroids by Dr. Pond and the

œstrogens and progesterone were recommended by Joslin and White from the 16th week of pregnancy, but this did not entirely prevent the accidents in pre-diabetic pregnancies. It is a fact, however, that chorionic gonadotrophin may kill the fœtus, or that when the pregnancy goes beyond term the fœtus may be overweight.

Hullquist and Engfeldt (1949) have produced giant new-born in rats when growth hormone was injected; Watts (1935) has reported the same effect, but Young (1949) has found resistance against diabetogenic effects of growth hormone during pregnancy

Two points are known about the last weeks of pregnancy —

1. The excretion of glycocorticoids is high (Venning and Browne, 1950; Cope, Boysen and McCrae, 1951).

2. Recently Bush has seen that there is a high level of 11-oxysteroids in the blood of pregnant women

We have taken up the study of the effects of cortisone on pregnant rabbits. Houssay (1945), Courrier (1950) and Robson and Sharaf (1952) discovered that cortisone and ACTH interrupt pregnancy after a few days. The dosage they used was very high, varying between 10 to 25 mg. for rabbits of 3 kg, and 2 mg. for mice.

P. L Hoet in his experiments estimated the placental glycogen and the effects of cortisone. The date of pregnancy and the dosage of cortisone have to be considered carefully as these will change completely the picture of results.

Experiments

A. *Group* 1—Three rabbits, 17 or 18 days pregnant, were injected with either 2 or 5 mg of cortisone per kg per day, and killed either 3 or 4 days later. The results are shown in Table I.

It will be seen that of the three rabbits, in one all the fœtuses were alive; in the other two, all were dead, and reabsorption of the uterine contents started immediately, as can be seen from reduction in weight of the fœtus.

PREGNANCY AND DIABETES

J. P. HOET

It is now known that in a diabetic pregnancy the dosage of insulin has to be increased throughout, but that as soon as the birth takes place there is a marked tendency for hypoglycæmia to occur (MacLeod, 1926; Lawrence and Oakley, 1942). In some cases, insulin dosage has to be reduced to a fourth of that tolerated during pregnancy.

Clinical observations show that the babies of a treated diabetic are frequently born sluggish, cyanosed and with an irregular respiration Also, in diabetic pregnancy there is a high incidence of prematurity, miscarriage and intra-uterine death: even with the most careful control, the "fœtal loss rate" remains high (Miller, Hurwitz and Kudder, 1944; Gilbert and Dunlop, 1949)

Such accidents in pregnancy occur in women who later become diabetics, long before permanent glycosuria is present. In pre-diabetes also, an increasing size of the babies is reported in women of all ages (Kriss and Futcher, 1948). If all the anomalies of pregnancy are taken together—large babies included—80 per cent of diabetic pregnancies are abnormal, and this can be traced back two to five years before the diagnosis of diabetes has been established.

It is, of course, highly probable that transitory disturbances of carbohydrate metabolism may exist years before full diabetes is recognized (Allen, 1939: Hurwitz and Jensen, 1946). Paton (1948) has demonstrated that pregnancies are abnormal 35 years before the clinical diagnosis of diabetes.

Hyperglycæmia—or a transitory reduction of tolerance for dextrose—is another manifestation in pre-diabetic pregnancy, (Hurwitz and Jensen, 1946; Verhaegen and Bijvoet, 1950). Its possible physiological explanation has been that there is a high level of chorionic gonadotrophin, and large doses of

increase in placental weight, but the fœtus remained under 1·g. until the 18th day.

When we gave ½ mg./kg./day of cortisone from the 10th day, an increase in glycogen of the placenta, both *fœtal and maternal part*, resulted (Table III) The

Table III

EFFECT OF CORTISONE ON PLACENTAL GLYCOGEN
(14th day of pregnancy)

Rabbit	*Dose of cortisone daily for 4 days in mg/kg/day*	*Number of fœtuses*	*Average weight of fœtuses*	*Weight of placenta in g*		*Glycogen content of placenta in g %*	
				maternal part	*fœtal part*	*maternal part*	*fœtal part*
L55	½ mg	14	0 28	1 32	0 46	6 24	2 74
L56	½ mg	14	0 23	0 96	0 45	4 98	1 81
L28	½ mg	8 (all dead)		0 79	0 66	5 8	0 42

figures of 6 24 g. and 4 98 g. of glycogen per 100 gr. placenta are very high Still more significance must be given to the proximal placenta whose glycogen content reached 2 74 per cent glycogen content.

The serum of the pregnant animals under cortisone (½ mg./kg /day for 4 days) was very opalescent.

Conclusions

1. Whatever may be the rôle of growth hormone and chorionic gonadotrophin in the abnormal pregnancies of pre-diabetics, an increase in 11-oxysteroids during pregnancy affects the nutrition of the fœtus and eventually produces miscarriage and death of the fœtus

2. Cortisone in doses of 2 mg /kg /day interrupts pregnancy in the rabbit. In doses of ½ mg./kg /day, it improves the rate of weight increase of the fœtus. Cortisone increases the placental glycogen up to the 18th day of pregnancy.

Table I

EFFECT OF CORTISONE ON PREGNANCY AND PLACENTAL GLYCOGEN
(21st day of Pregnancy)

Rabbit	*Dose of cortisone in mg/kg/day*	*Days before killed*	*Number of fœtuses found*	*Average weight of fœtuses*	*Placental weight in g*		*Glycogen content of placenta in g %*	
					maternal part	*fœtal part*	*maternal part*	*fœtal part*
L42	2	3	9 (all alive)	3·40	1·25	1·02	1·48	0·34
L44	2	4	9 (all dead)	1·90	1·33	0·94	0·22	0·18
L43	5	3	8 (all dead)	0·85				

Group 2—½ mg. cortisone/kg./day was given to a rabbit from the 16th to the 19th day of pregnancy; another rabbit of the same stock was mated at the same time and killed also on the 19th day of pregnancy. Fortunately, the rabbit to whom the cortisone was given *had 12 fœtuses, the control had 7. The average* weight of the fœtuses of the former rabbit was 2·85 g and the placental weights and their glycogen content less than that of the control (Table II).

Table II

EFFECT OF CORTISONE ON WEIGHT OF FŒTUSES AND PLACENTAL GLYCOGEN
(19th day of pregnancy)

Rabbit	*Dose of cortisone daily for 4 days in mg/kg/day*	*No. of fœtuses*	*Average weight of fœtuses*	*Weight of placenta in g*		*Glycogen content in g %*		
				maternal part	*fœtal part*	*maternal placenta*	*fœtal placenta*	*fœtal liver*
L51	½	12	2·75	1·34	1·38	0·475	0·288	0·187
L52	—	7	2·12	2·03	2·01	0·81	0·25	0·18

B. To follow the other set of experiments, I shall refer to the graph drawn by Hammond (reproduced in Marshall's Physiology of Reproduction (1952) vol. II, 313) on the growth of uterine contents during pregnancy. Up to the 16th day of pregnancy there was a slow

DISCUSSION

MANN Regarding diabetic pregnancy, it would seem to me that some useful information could be obtained by chemical studies on both the maternal and the fœtal blood and fluids. As shown first by Claude Bernard, allantoic and amniotic fluids contain fructose, and that observation has been confirmed and extended by Carl Neuberg We also know that sheep fœtal blood contains a mixture of glucose and fructose. This was conclusively shown by Bacon and Bell in Cambridge. Huggett has shown at St. Mary's Hospital medical school in London that infusion of glucose into the maternal circulation influences the level of fructose in the fœtal circulation. Another substance which may be worth while investigating is inositol, because it is known that inositol is excreted in large quantities in the urine of diabetics, and in some species, particularly in certain rodents, an inositol deficiency causes abortion

GRAY. I should like to comment on Dr Hoet's remarks about Compound F in pregnant women's blood Our patients were either perfectly normal pregnancies and not toxæmic, or else were diabetic pregnant patients Two out of the seven had either a fœtal death or a neo-natal death I understood that Bush has shown massive amounts of Compound F in the peripheral blood in patients with very severe pre-eclamptic toxæmia I wondered whether that point would explain the apparent discrepancies between our results and the ones to which Dr Hoet referred It emphasizes once again the point that pre-eclamptic toxæmia is a different condition from the ordinary problems of diabetic pregnancy

HOET: When I saw the chromatograms of Bush, they were not abnormal pregnancies I think in normal pregnancy, at 7 or 8 months,

may be twice as high in the serum, I don't think that should mean that normal pregnancy has a diabetogenic action which plays on some established function of the pancreas. I think therefore that there is nothing very different between a normal pregnancy and a diabetic pregnancy, except that the diabetic pregnancy is one coming in a person where you can study the sugar in the urine very easily If you took ordinary
two overweigh
even in their p
the baby I g
diabetic pregn
5 4 mg I couldn't tell therefore whether there is an abnormal figure of corticoids in the urine in diabetic pregnancies I think that we must study the people 30 years before they get plain diabetes. That is very

3. The increase of 11-oxysteroids in the blood of pregnant animals will play an important part in the diabetogenic effects of pregnancy. The degree of hyperglycæmia will depend on the functional value of the islets of Langerhans

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sugar rises fairly high, tends to stay high and does not return as rapidly as it should; some of these patients become quite normal after delivery There is a third group which start off like the second group, but after delivery their tolerance curve is worse, that is the blood sugar tends to go still higher and takes still longer to come down, and it is perhaps in this group that we may expect to see something happening in later years. We would like to be able to follow up these women and maybe we'll have enough left in 10 years' time to be able to say what has become of them.

MIRSKY. That's selecting them on the basis of the glycosuria?

SWYER: Yes, on the basis of glycosuria

CONN. We've done it on unselected normal pregnant women, in

to hormonal activities in complicated pregnancies which hasn't been brought up yet. It concerns the possible rôle of the liver in the con-

hepatitis that the excretion of 11-oxy-steroids in the urine is much higher than in normals, and that the excretion of 17-ketosteroids is lower This suggested to us the possibility that the 11-oxy-steroids produced were not being converted to 17-ketosteroids for excretion

when the material is given by mouth It must be assumed that the liver plays an important rôle in the metabolism of steroids In toxæmia and in diabetic pregnancies the liver may be functionally inadequate in this respect

YOUNG Reverting to the question of the induction of diabetes in

YOUNG I have speculated at various times that under those conditions the growth hormone was able to bring about a retention of nitrogen which would assist fœtal development, the corollary of this is that

KOLLER: If I understood you correctly, you think that the so-called idiogravidic glycosuria might be explained by the increase of corticoids during pregnancy? How do you explain the normal or even subnormal sugar tolerance curve so often found in cases with glycosuria of pregnancy?

[illegible]

error of the blood sugar curve that in 100 per cent of cases it was abnormal; Modiyoms and Frank with 100 g. of sugar found sugar in the urine in 98 per cent of cases, very often starting with a very low figure. I remember Conn has been telling us that there is renal glyco-

[illegible]

functional condition of the pancreas.

MIRSKY: We have utilized a clinical observation as a method for selecting a population with a high incidence of potential diabetes

[illegible]

had developed it by the time we saw the group, we studied women who had given birth to their babies within 48 hours. Again, we could find no trend in the distribution of sugar tolerance curves that would indicate that we were dealing with two different populations as we expected, since approximately 40 per cent of the women with overweight babies will develop diabetes in the future. We tested the patients within two days—I didn't think it was quite fair to go to the mother before that.

HOLT: You know that the corticoids fall to 1/4 about 5 days after

[illegible]

stays normal after delivery. There is another group where the blood

labour in many people. It is not a uniform occurrence, but I should think occurs in 50 per cent of them. I have seen a woman who has been having 40 units a day for 15 years come off insulin entirely for a month

OAKLEY: There are two points which have been passed over. One is this, that although as pregnancy proceeds the insulin requirement in about 70 per cent of cases increases in a significant number it falls [illegible] de-

r cent

Hoet

lin in

pregnancy, resulted in the mother having a live baby after she had had 4 or 5 miscarriages. Gilbert and Dunlop and others have shown that the incidence of fœtal loss is the same, or comparable, in insulin and non-insulin diabetics—the severity of the diabetes apparently plays no part in the fœtal loss rate Furthermore, the most significant and peculiar thing about the fœtal loss rate is that it is almost as high now as it was before insulin was discovered, the great change being in the *maternal* loss rate which has been improved so much by insulin The fœtal loss rate has not been very greatly changed by insulin If you look over a big survey in pre-insulin days, you can confirm this—in mothers who weren't moribund or dying at the time, the amazing thing is that the fœtal loss rate was so little different then from what it is in many places now.

BEST That is the rate, and not total number of survivals?

OAKLEY· Well, if you take the rate in a sufficient number of people it becomes comparable, doesn't it?

HOLT I should like to say that what Gilbert and Dunlop have noted about insulin or non-insulin cases is that if you take the patients—diabetic women of 45 or 55—and they have sugar in their urine, then you ask them if they have had overweight babies, miscarriages, or [illegible]

cause the disappearance of diabetes on earth. That may not be so but it is definitely a rational possibility. But I don't believe it

the off-spring should be larger under those conditions. There are various claims in the literature, particularly with regard to the rat, that such is true, but we've not been able to get any clear-cut data on that point.

LAWRENCE. We've seen a good many diabetic pregnancies in our department, and we've done a fair amount of work on glucose tolerance tests in normal diabetic pregnancies I've read a good many contradictory papers On the whole I haven't seen a clear preponderance of pre-diabetic curves in ordinary pregnancies There are one or two other points I might mention. I think I should say that we've never seen diabetes, however mildly it started in pregnancy, disappear or get cured afterwards, and I don't think it was from lack of treatment—when they needed insulin, they got insulin. We've never had that good fortune.

HOLT. The case of Verhaegen needed 80 units and came back to normal glycæmic figures after childbirth. I have come across cases where with 10 to 30 units, normal babies were born in persons who had had 3 or 4 fœtal deaths before without any clinical history of diabetes Since they had slightly abnormal sugar tolerance curves we gave insulin and the birth of the child was normal, it was a heavy baby but it was [illegible]

tolerance curve means in pregnant organisms is quite a different thing from what it means under normal circumstances. With those authors who never saw any difference—I forget their names now—it was probably because they only saw one or two independent curves If instead they had done them periodically, or 4 or 5 times at least during pregnancy, they would have found the difference, I am sure

MIRSKY: I think that is an important point, because if there is an increase in glycosuria in some of these instances, it may well be that the absorption of sugar directly from the stomach may be increased under such circumstances. We had some experiences which indicated that that may occur with corticoid administration I would like to clear up one point with reference to the sugar tolerance curves that I mentioned In a population in which you can anticipate that about 40 per cent will develop diabetes at any time up to 20 years after the birth of the baby, one would anticipate that within 48 hours after [illegible]

single population.

LAWRENCE: I think, Dr. Mirsky, that your procedure might be criticized in that you [illegible]
when their corticoids [illegible]
rebound from pregnan[illegible]
for the curious reduct[illegible]

AUTHOR INDEX

Plain numbers indicate a contribution by the author, either in the form of a paper or to the discussions

Numbers in italics indicate a reference to the author's work

SUBJECT INDEX

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